Characterisation of HLA-restricted T-cell responses to abacavir using lymphocytes from drug-naïve volunteers

This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

Catherine Clare Bell
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Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree

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Catherine C. Bell

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Contents

Abbreviations

A_{260}	Absorbance at 260nm
ABC	Abacavir
ADH	Alcohol dehydrogenase
ADR	Adverse drug reaction
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
APC	Allophycocyanin
ARDS	Acute respiratory distress syndrome
β_2m	β_2 microglobulin
BE	Bullous exanthema
BSA	Bovine serum albumin
CCR	Chemokine receptor (C-C) motif
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CI	Confidence interval
cpm	Counts per minute
CRM	Chemically reactive metabolite
CSA	Cyclosporin
C_t	Cycle threshold
CTL	Cytotoxic T-lymphocyte
CV	Coefficient of variation
CYP	Cytochrome P450 enzyme
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DHR	Drug hypersensitivity reaction
DILI	Drug-induced liver injury
DMSO	Dimethyl sulfoxide
DNA	Dioxyribonucleic acid
DNCB	Dinitrochlorobenzene
DNP	Dinitrophenol
DRESS	Drug reaction with eosinophilia and systemic symptoms
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme-linked immunospot
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
FBS	Foetal bovine serum
FDA	Food and drug administration (US)
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GrzB	Granzyme B
GSH	Reduced glutathione
h	Hours

HBSS	Hanks balanced salt solution
HCP5	HLA complex P5
HEPES	hydroxyethyl piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Hm	Homozygote
HMGB1	High-mobility group box 1
Hsp70	Heat shock protein 70
HSS	Hypersensitivity syndrome
Ht	Heterozygote
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IL	Interleukin
IS	Internal standard
ITAM	Immunoreceptor tyrosine-based activation motifs
JNK	c-Jun N-terminal kinase
kB	kilo base
LAT	Transmembrane adapter protein linker for the activation of T-cells
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LPS	Lipopolysaccharide
LSD	Lycergic acid diethylamide
LTT	Lymphocyte transformation test
Mb	Megabase
ME	Maculopapular exanthema
MHC	Major histocompatibility complex
min	Minutes
MRM	Multiple reaction monitoring
NAD	Nicotinamide adenosine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	not determined
NFAT	Nuclear factor of activated T-cells
NHS	National health service
NK	Natural killer
NP	Not performed
NRTI	Nucleoside reverse transcriptase inhibitor
OR	Odds ratio
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Papular exanthema
PE	Phycoerythrin
pH	Power of hydrogen
pi	Pharmacological interaction
pKa	Acid dissociation constant
PKC	Protein kinase C

PΩ	C-terminal anchor residue
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
R_t	Retention time
SFC	Spot forming cell
SI	Stimulation index
SJS	Stevens-Johnson syndrome
SLP-76	SH2 domain-containing leukocyte phospho-protein of 76kDa
SMX	Sulfamethoxazole
SMX-NHOH	Sulfamethoxazole hydroxylamine
SMX-NO	Nitroso sulfamethoxazole
SNP	Single nucleotide polymorphism
SSOPH	Sequence specific oligonucleotide probe hybridization
SSP-PCR	Sequence specific primer polymerase chain reaction
STAT	Signal Transducer and Activator of Transcription
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TEN	Toxic epidermal necrolysis
Th1	Type 1 helper cell
TNCB	Trinitrochlorobenzene
TNF-α	Tumour necrosis factor-α
TNP	Trinitrophenol
TPMT	Thiopurine methyltransferase
tSNP	Tagging single nucleotide polymorphism
UDP	Uridine diphosphate
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UK	United Kingdom
ULN	Upper limit of normal
v/v	volume/volume
VKORC1	Vitamin K epoxide reductase complex subunit 1
w/v	weight/volume
WHO	World Health Organisation
ZAP-70	ζ-chain-associated protein kinase 70

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Understanding, predicting and preventing serious adverse drug reactions in man using *in vitro* models

Alternatives to Laboratory Animals

Abstract

Immune-mediated adverse drug reactions are difficult to predict and can be severe in nature. Recently observed genetic associations highlight the importance of specific human leukocyte antigen alleles in the development of certain reactions. The mechanisms underlying antigen formation and subsequent T-cell activation require further investigation. The drugs abacavir (HLA-B*57:01) and ximelagatran (HLA-DRB*07:01 and HLA-DQA*02:01) represent compounds associated with skin and liver reactions respectively, for which a HLA association has been reported.

In order to investigate the mechanism of HLA-restricted T-cell activation a cohort of 400 healthy volunteers was established. Both functional lymphocytes and DNA were isolated and stored. Following sequence-based HLA-typing twenty-six individuals expressing HLA-B*57:01 were identified (1 homozygote, 25 heterozygotes) and 101 individuals (10 homozygotes, 91 heterozygotes) expressing HLA-DRB*07:01 were identified.

T-cells from these volunteers were utilised in *in vitro* assays. The first assays employed had low sensitivity and were unable to detect any drug-specific T-cells either by proliferation or cytokine secretion. Seventy-four $CD8^+$ abacavir-specific T-cell clones however were generated from 3/3 volunteers expressing HLA-B*57:01. These clones secreted an array of cytokines and cytotoxic mediators (IFN-γ, Granzyme B, perforin, Fas ligand) in response to drug incubation.

Chemically reactive metabolites are frequently associated with adverse drug reactions. The metabolism of abacavir in both liver and immune cell preparations was therefore assessed. In human liver cytosol, abacavir was metabolised to three isomeric carboxylic acids (48±15% turnover at 20h). This reaction proceeded via a reactive aldehyde metabolite that could be trapped with methoxylamine. Metabolism was blocked by the addition of 4-methylpyrazole, an alcohol dehydrogenase inhibitor (1000μM = 91.7±3.9% inhibition; $p<0.05$). Low-level carboxylic acid formation could be detected in an S9 fraction (2%) and cytosol (3.6%) generated from antigen-presenting cells suggesting that T-cell clones are exposed to a small amount of the aldehyde metabolite during cellular assays. The parent drug however is the predominant chemical entity present.

Cross-reactivity at the MHC-TCR interface was determined with a number of abacavir analogues. The interaction was found to be highly specific with only deuterated abacavir stimulating T-cell clones at a similar level to abacavir. Dihydro abacavir only stimulated clones when used at high concentrations. The alternative enantiomer of abacavir (1S,4R) and carbovir did not stimulate any clones at any concentration investigated.

This work further highlights the involvement of T-cells in drug hypersensitivity reactions and the importance of previously described HLA associations. In addition, chemical restriction exists at the HLA-TCR interface and is key to the activation of abacavir-specific T-cell clones. The metabolism of abacavir to a protein-reactive metabolite can occur in antigen-presenting cells therefore the potential of reactive intermediates to activate T-cells in a HLA-restricted manner should be investigated.

Chapter One

General Introduction

1.1 Introduction

Immune-mediated reactions to drugs are difficult to predict and can be extremely severe in nature. Recent genome-wide association studies have reported increased susceptibility to adverse drug reactions with the possession of specific human leukocyte antigen (HLA) alleles. The evidence for the involvement of drug-specific T-cells in the pathogenesis of many of these reactions is well established however; the molecular basis of these HLA associations is not fully understood.

1.2 Adverse Drug Reactions

1.2.1 Definitions

In 1972, the World Health Organisation (WHO) defined an adverse drug reaction as follows:

"A response to a drug that is noxious and unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function" (WHO, 1972)

Though still relevant, a more comprehensive definition has been proposed by Edwards and Aronson (2000):

"An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen"

The authors suggest that the latter definition is more useable as it refers to serious reactions, includes reactions caused by contaminants or excipients and includes reactions caused by prescription error.

1.2.2 Impact and prevalence

Adverse drug reactions (ADRs) represent a major clinical problem and a serious impediment to the advancement of new therapeutic agents. Despite rigorous pre-clinical and clinical testing strategies, approximately 3% of new compounds will ultimately be withdrawn from the market due to safety concerns, particularly in the first two years following approval (Lasser et al., 2002).

The incidence of ADRs and their associated severity identifies them as a significant health issue worthy of investigation. In 2004, a prospective study of over 18,000 hospital admissions in the North-West of England found that approximately 6.5% were attributable to an adverse drug reaction (Pirmohamed et al., 2004). Reactions were particularly prevalent in the elderly (median age in ADR group = 76; median age in non-ADR group =66) and amongst women (59% in ADR group; 52% in non-ADR group) (Pirmohamed et al., 2004). This is consistent with other studies in which data from multiple centres in the UK was collated (incidence of ADRs 12.9 per 10 000 patient months for males vs. 20.6 per 10 000 patient months for females)(Martin et al., 1998). In the majority of cases patients recovered from their ADRs, however 2.3% of ADRs were classified as being directly responsible for patient fatality

(Pirmohamed et al., 2004). When this was put in the context of the cause of death among all hospital patients, ADRs were associated with fatalities of up to 0.2% (Lazarou et al., 1998, Pirmohamed et al., 2004). Based on this estimate Lazarou et al. suggest that ADRs therefore represent between the fourth and sixth leading cause of death in the US (Lazarou et al., 1998).

In addition to the direct risk to patient safety, ADRs have serious economic consequences. Pirmohamed et al. estimated that ADRs cost the NHS more than £400 million per year, representing a significant financial burden (2004). Additionally, pharmaceutical companies stand to lose substantial investment following late stage withdrawal of a compound. Various estimates put the financial cost of bringing a new drug to market at up to $800million (DiMasi et al., 2003).

1.2.3 Classification

Serious adverse drug reactions have been reported for a diverse array of drugs with varied indications and frequencies (Table 1.1). These reactions can largely be grouped in to two major categories; type A, on-target reactions and type B off-target reactions (Park et al., 1998).

Type A (on-target) reactions are generally a function of dose, causing an exaggerated pharmacological effect. They may be influenced by metabolism, drug transporters or enzyme induction/inhibition by co-administered therapies. In most instances it is possible to control these types of reactions by careful therapeutic monitoring or through dose adjustment based on the patient's genetic background. Type A reactions represent the majority of ADRs. Type B (off-target) reactions, though less frequent are a leading cause of drug

attrition due to their severe and unpredictable nature. Effects are unrelated to pharmacological action and affect only a small proportion of susceptible individuals. Given these off-target effects, it can be very difficult to unravel the underlying mechanisms.

A small number of ADRs fall outside of these classifications and so the following definitions may therefore also be relevant (Edwards and Aronson, 2000):

- Type C- Chronic

  These reactions are associated with long term use of a particular drug e.g iatrogenic Cushing's syndrome caused by raised glucocorticoid levels during treatment with prednisolone.

- Type D – Delayed

  These reactions may become apparent years after the end of treatment. For example secondary tumours associated with the use of alkylating agents. This definition also applies to teratogenic drugs causing adverse effects to the offspring of mothers undergoing drug treatment while pregnant e.g thalidomide and valproate are associated with birth defects.

- Type E – End of Use

  Occur upon withdrawal of the drug. In some cases they may be avoided by a gradual reduction in dose. For example, sudden withdrawal of β-adrenoreceptor antagonists is associated with unstable angina whereas withdrawal of anticonvulsants can result in seizures.

Despite stringent *in vivo* and *in vitro* testing procedures, unsafe drugs are still reaching the market. A greater understanding of the mechanisms underlying known ADRs is therefore required in order to highlight potential areas for improved screening of new chemical entities.

Table 1.1 Examples of adverse drug reactions

Drug	Indication	Adverse reaction	Frequency in study population	Date withdrawn	Reference
Abacavir	HIV	Hypersensitivity	5-8%	Still in use +	(Mallal et al., 2002)
Carbamazepine	Epilepsy	SJS/TEN	0.1-0.01%	Still in use +	(Vittorio and Muglia, 1995)
Cerivastatin	Cholesterol lowering	Rhabdomyolysis	<0.1%	2001	(Furberg and Pitt, 2001)
Cisapride	Gastric reflux	Cardiotoxicity	1.5%	2000	(Yap and Camm, 2003)
Clozapine	Schizophrenia	Agranulocytosis Cardiotoxicity	1%	Still in use	(Alvir et al., 1993)
Isoniazid	Tuberculosis	Hepatotoxicity	10-20%* 1%§	Still in use	(Mitchell et al., 1976)
Rifampicin	Tuberculosis	Hepatotoxicity	<5%*	Still in use	(Grosset and Leventis, 1983)
Rofecoxib	Arthritis	Heart attack/stroke	3%	2004	(Bresalier et al., 2005)
Troglitazone	Diabetes	Hepatotoxicity	1.9%*	2000	(Watkins and Whitcomb, 1998)
Warfarin	Anti coagulant	Haemorrhage/ Bleeding	10-16%	Still in use	(Wysowski et al., 2007)
Ximelagatran	Thromboembolism	Hepatotoxicity	7.9%*	2006	(Lee et al., 2005)

+ genotyping recommended * elevated ALT § overt hepatotoxicity

1.2.4 Drug-induced liver injury

Drug-induced liver injury (DILI) is a rare but serious side effect and is a leading cause of withdrawal of newly developed drugs from the market. Identifying potentially hepatotoxic drugs during the development process can be difficult due to limitations in the experimental systems available and the lack of predictability of pre-clinical animal models (Kaplowitz, 2005). This is especially relevant where the immune system is thought to be involved.

Idiosyncratic adverse reactions occur in only a small number of susceptible individuals at a frequency thought to range between 1 in 10 000 and 1 in 100

000 of treated patients (Holt and Ju, 2006). Consequently many of these reactions cannot be detected during the development process and only become apparent when the drug is released to the wider population. This makes DILI a major cause of post-licensing labelling restrictions and drug withdrawal (Watkins, 2005). Careful monitoring is required, as liver injury may not be apparent until weeks or months after the commencement of therapy and is not always confined to one area of the liver (Wilke et al., 2007).

During clinical trials a number of readouts are monitored in order to ensure the safety of participants. Monthly measurements of serum liver enzymes, in particular alanine aminotransferases (ALTs), are performed routinely. These enzymes are released passively from dying hepatocytes. If mild elevations are recorded (i.e. 3 x ULN) sampling may be performed more frequently and severe elevations (i.e. 10 x ULN) are justification for immediate termination of the trial (Lee and Senior, 2005). This can however mean that a trial is stopped before any overt symptoms of liver injury are actually observed and therefore the true hepatotoxic potential of the new drug may not be fully established. The phenomenon of adaptation can also confound the situation as in some instances elevations in ALTs are transitory and resolve despite continuation of the drug (Kindmark et al., 2008). The mechanisms responsible are not widely understood, although the down regulation of metabolic enzymes has been implicated in adaptation to repeated doses of paracetamol (Shayiq et al., 1999). It may also be the case that adaptation is dependent on the individual (Au et al., 2011).

Measurement of serum ALTs does not however give any indication of impaired liver function. For this reason bilirubin levels are commonly measured alongside ALTs. A build up of bilirubin in the serum indicates that haem metabolism has been disrupted. Much smaller fluctuations in bilirubin levels are tolerated in clinical trials (>2 x ULN), reflecting the severity of this side effect. The combined appearance of both jaundice (bilirubin >2 x ULN) and heaptocellular injury (ALT > 3 x ULN) is the basis of Hy's law, which estimates that these cases are associated with 10-50% mortality (Zimmerman, 1999).

Other tests used clinically include prothrombin time, which is a measure of the time taken for patient plasma to clot. A prolonged prothrombin time (INR ≥ 1.5) may be indicative of reduced clotting factor synthesis and is observed during acute liver failure. At this stage it may be necessary to consider liver transplantation (Reuben et al., 2010).

Following observation of abnormal liver tests it can still be difficult to diagnose DILI and to determine causality. The number of patients experiencing a reaction is generally low and so it must be confirmed that the drug is responsible and not another underlying disease state. A number of 'hallmarks' of DILI can be used to aid diagnosis, these include: time of onset (within a few weeks of beginning drug therapy), an easing of the reaction when the drug is stopped, and a more severe and rapid reaction upon rechallenge (Lee and Senior, 2005). Diagnosis can however be confounded by the use of multiple drugs, hepatitis infection and congestive heart failure. Liver tests may also be abnormal in obese or diabetic patients.

1.3. Immunological Reactions

Type B reactions can involve the immune system. ADRs with an immunological aetiology tend to be severe and are incredibly difficult to predict since there are few preclinical screens capable of recapitulating the complex nature of the immune system either *in vivo* or *in vitro*. These reactions will be the focus of the rest of this thesis.

1.3.1 Classification of immunological reactions

Drug hypersensitivity can be defined as a serious adverse drug reaction with an immunological aetiology to an otherwise safe and effective therapeutic agent. Gell and Coombs (1963) first categorised immunological reactions into four groups based on the key clinical features and type of immune response. Types I-III represent antibody-mediated reactions, whereas type IV reactions are caused by T-cells. This classification is still widely in use today, with the further division of type IV reactions introduced more recently (Pichler, 2003). The key features of each type of reaction are summarised in Table 1.2. It must however be noted that a great deal of overlap is often observed, particularly with regard to the specific mediators released. Delayed-type T-cell mediated reactions (Type IV) will be discussed further in this thesis.

Table 1.2 Extended classification of immune-mediated adverse drug reactions.

	Type of immune response	Pathologic characteristics	Clinical symptoms	Drug binding	Cell Type
Type I	IgE	Mast cell degranulation	Urticaria and anaphylaxis	Covalent	B-cells
Type II	IgG and FcR	FcR dependent cell destruction	Blood cell dyscrasia	Covalent	B-cells
Type III	IgG and complement or FcR	Immunocomplex deposition	Vasculitis	Covalent	B-cells
Type IVa	Th1 (IFN-γ)	Monocyte activation	Eczema	Covalent/non-covalent	T-cells
Type IVb	Th2 (IL-5, IL-4)	Eosinophilic inflammation	ME, BE	Covalent/non-covalent	T-cells
Type IVc	CTL (perforin, granzyme B)	$CD4^+$ or $CD8^+$ cytotoxicity	ME, eczema, BE and PE	Covalent/non-covalent	T-cells
Type IVd	T-cells (IL-8)	Neutrophil recruitment and activation	PE	Covalent/non-covalent	T-cells

ME = Maculopapular exanthema, BE = Bullous exanthema, PE = Pustular exanthema. Adapted from (Pichler, 2003)

1.3.2 Clinical Manifestations

Skin is the most frequent site of hypersensitivity reactions. The severity of symptoms depends on both the chemical agent administered and the individual. The majority of cutaneous ADRs (>90%) are benign exanthematous or maculopapular eruptions (Hunziker et al., 1997) which first appear on the trunk and upper extremities, before becoming widespread (Roujeau, 2005). They usually begin 4-14 days after beginning drug treatment, although sometimes may not appear until shortly after treatment has finished (1-2days).

Drug reaction with eosinophillia and systemic symptoms (DRESS) is a more severe side effect. It is associated with 1 in 10, 000 exposures to drugs such as antiepileptics (e.g lamotrigine) and sulfonamides (e.g sulfamethoxazole)

(Roujeau, 2005). As the name suggests, this reaction commonly affects multiple organs including liver, kidney and lungs (Guillon et al., 1992). An increase in circulating eosinophils is characteristic and may coincide with an increase in circulating lymphocytes. Onset is generally later than other cutaneous reactions and may not be observed until 2-6 weeks after first administration. DRESS has a mortality rate of about 10% (Roujeau, 2005), which can often be as a result of liver involvement. Viral reactivation is often observed during DRESS and can be used as a diagnostic criterion (Suzuki et al., 1998, Tohyama et al., 1998). It is currently unclear what role this reactivation has in the pathogenesis of the disease and whether it is a cause or consequence of the clinical symptoms. Reactivation of human herpes virus 6 (HHV-6) is measured by increases in specific IgG and viral DNA. This however is often not detectable until several weeks after symptoms have commenced (Shiohara et al., 2006). It is therefore possible that viral reactivation occurs as a result of T-cell activation. It is not just HHV-6 that has been implicated in DRESS. Transient increases in virus-specific immunoglobulins against cytomegalovirus (CMV), HHV-7 and Epstein-Barr virus (EBV) have also been observed (Aihara et al., 2001, Descamps et al., 2003). These elevations are thought to occur in a sequential manner though this order is not universal in all patients (Kano et al., 2006). Cells taken from skin lesions have been found to contain high levels of HHV-6 DNA and express viral antigens at an early timepoint (Suzuki et al., 1998). Clinical manifestations could therefore be caused by the expansion of virus-specific T-cells (Shiohara et al., 2006).

Stevens-Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) are the most severe cutaneous adverse drug reactions. Blistering skin lesions are characteristic. It is now widely accepted that the two are in fact a spectrum of the same disease, with the degree of skin detachment being used to delineate them (Mockenhaupt, 2011, Bastuji-Garin et al., 1993). Histopathology reveals that death of keratinocytes in the epidermis is initially caused by apoptosis though this eventually leads to necrosis and skin detachment (Paul et al., 1996). Skin lesions contain high numbers of NK and cytotoxic T-cells suggesting they are the effector cells (Chung et al., 2008). Analysis of blister fluid has revealed granulysin to be a key soluble mediator, the levels of which appear to correlate with clinical severity (Chung et al., 2008). Measurement of granulysin in patient serum may therefore serve as an early diagnostic test (Abe et al., 2009). Fatality rates are high, approximately 10% in SJS and more than 30% in TEN. Death often occurs as a result of sepsis or pulmonary symptoms. Treatment may require the expertise of a specialist burns unit. Viral reactivation is occasionally associated with SJS/TEN though is not a defining feature (Teraki et al., 2010). Table 1.3 summarises the main features of DRESS and SJS/TEN.

Table 1.3 Characteristics of common cutaneous adverse drug reactions.

	Cutaneous drug reaction		
Symptom	SJS	TEN	DRESS
Mucous membrane	>90%	>90%	<30%
Erosions	Several sites	Several sites	Mouth and lips
Detachment of epidermis	Yes <10% of BSA	Yes >30% of BSA	No
Hyperkeratosis/desquamation	No	No	Usual
Neutropenia	No	30%	No
Eosinophilia	No	No	90%
Atypical lymphocytes	No	No	30-40%
Respiratory tract	Bronchial erosions/ARDS	Bronchial erosions/ARDS	Interstitial pneumonitis
Liver	Hepatitis 10%	Hepatitis 10%	Hepatitis 60%
Heart	No	No	Myocarditis
Lymph node enlarged	No	No	Usual
Viral reactivation	Occasional	Occasional	Frequent

SJS= Stevens-Johnson syndrome, TEN= toxic epidermal necrolysis, DRESS= drug reaction with eosinophilia and systemic symptoms, BSA= body surface area, ARDS= acute respiratory distress syndrome. Adapted from (Bachot and Roujeau, 2001)

1.4 Immune activation

1.4.1 Cells of the immune system

The immune response is comprised of both adaptive and innate immunity. Innate immunity is a non-specific, generalised response often as a result of tissue damage/trauma or infection. Adaptive immunity is an acquired response, which specifically recognises antigens. A vast array of cell types are involved in both types of immunity. For the most part, immune cells have a short life span and are constantly replaced. All cell types originate from pluripotent stem cells found in the bone marrow and depending on various stimuli, including cytokines and hormones, a number of phenotypically different cell types can be generated. They can be divided into the following classes each with a specialised function:

Lymphocytes

Lymphocytes constitute approximately 20% of the leukocytes found in blood. B, T and NK cell subsets are all derived from lymphoid progenitor cells but have unique functions and developmental lineages. B-cells initially develop in the foetal liver before migrating to the bone marrow for further development. The general function of B-cells is to produce and secrete antibodies. B-cells express immunoglobulins, which serve as antigen receptors. Following antigen encounter, B-cells will proliferate and differentiate into either plasma or memory cells. Plasma cells secrete antibodies with the same antigen specificity as the immunoglobulin displayed on the cell surface. These antibodies have several functions including the neutralisation or shielding of viral components, fixation of complement and opsonisation or coating of antigen in order to aid phagocytosis. A small number of long-lasting memory cells are also produced, which circulate in a resting state but respond rapidly when the antigen is encountered again. This is because memory cells express high affinity IgA or IgG molecules in contrast to the lower affinity Ig molecules expressed on naïve cells (Ig D and IgM). Experimentally, B-cells are identified by the expression of cell surface markers such as CD19. Specific IgE antibodies are associated with immediate hypersensitivity reactions, exemplified by the β-lactam antibiotics (Torres et al., 2003).

T-cells develop in the thymus. It is the T-cell receptor (TCR) that determines antigen specificity and each T-cell will only express one TCR hence only recognising a small number of antigens. In contrast to B-cells the TCR only recognises a small portion of the antigen, just a few amino acids. Upon exposure

to an antigen, antigen-specific T-cells bearing the corresponding TCR will divide and differentiate to give a large population of effector T-cells. Positive selection removes the vast majority (>95%) of newly developed thymocytes (Romagnani, 2006). T-cells expressing TCRs that have no affinity for MHC-peptide complexes die of neglect. Surviving cells then undergo negative selection which induces apoptosis in T-lymphocytes expressing TCRs which have a high affinity for self peptides found on the surface of medullary epithelial cells (Romagnani, 2006).

Subsets of T-cells express either CD4 or CD8 molecules. Originally this was thought to suggest the effector function of T-cells; $CD4^+$ T-cells were referred to as helper cells whereas $CD8^+$ T-cells were cytotoxic. However as some $CD4^+$ cells can also have cytotoxic functions this distinction is no longer relevant (Marshall and Swain, 2011). $CD4^+$ T-cells can also be divided according to the cytokines that they produce. Prior to antigen exposure, naïve cells are known as type 0 (Th0) cells. In the presence of IL-2 activated Th0 cells will become Th1 cells, whereas in the presence of IL-4 Th2 cells will be produced. Th1 cells subsequently produce IL-2 and IFN-γ whereas Th2 cells produce IL-4, IL-5, IL-9 and IL-13 maintaining differentiation down this lineage (Mosmann and Coffman, 1989). These different cytokine profiles then influence the type of immune response that occurs, Th1 cytokines promote cell-mediated immunity whereas Th2 cytokines promote a humoral (B-cell) response. Th17 cells are a relatively newly discovered subset of T-cells (Harrington et al., 2005) which are thought to have a role in diseases such as rheumatoid arthritis and multiple sclerosis (Tesmer et al., 2008). It is thought that IL-1β is a signal for T-cells to develop along this lineage (Acosta-Rodriguez et al., 2007).

Drug-specific T-cells, a hallmark of delayed hypersensitivity reactions, can be detected in the peripheral blood of hypersensitive patients and will be discussed later (see 1.5.5).

Natural killer (NK) cells make up a very small proportion of lymphocytes. They do not express antigen receptors but respond rapidly to viral infection and can also kill some tumour cells. Killing is mediated by perforins and granzyme molecules, which are secreted either in vesicles or directly. Previously the activation of NK cells was considered to be non-specific. However, they are now known to express a number of inhibitory and activating receptors. Inhibitory receptors such as killer-cell immunoglobulin-like receptors (KIRs) are specific for a number of ligands including MHC class I molecules. Upon encountering the corresponding ligand tyrosine phosphatases are recruited to the receptor, resulting in the dephosphorylation of proteins which have previously been phosphorylated by tyrosine kinases recruited to nearby activating receptors (Lanier, 2008). It is therefore the balance between the occupation of these receptors that determines the activation state of the NK cell. Activating receptors include DAP-12, NKG2D and CD244 (Lanier, 2008). It is thought however that a combination of activating signals are required in order to induce NK activation and not a single ligand-receptor interaction (Bryceson et al., 2006). Cell surface expression of CD56 is commonly used as a marker to identify NK cells *in vitro*.

Phagocytes

The main function of phagocytes is to engulf pathogens such as bacteria and subsequently break them down. This can be achieved in a number of ways and the different subsets of phagocytes will briefly be discussed here.

Monocytes develop from a myeloid progenitor cell. They circulate in the blood before migrating into tissues and differentiating into macrophages. They have large numbers of lysosomes, which contain digestive enzymes that break down pathogens. *In vitro*, they are identified by the presence of CD14. Once matured, macrophages generally reside in tissues, however a small proportion will return to the circulation where they are also capable of presenting antigens to T-cells. Kupffer cells are a specialised liver-resident macrophage.

Neutrophils make up the majority of leukocytes found in blood (approximately 70%). They are relatively short lived in the circulation and migrate to the tissues under the influence of chemoattractants such as IL-8. They are a granular cell type containing several different types of granule. These include acidic lysosomes and primary, secondary and tertiary granules containing molecules such as lactoferrin and myeloperoxidase. Neutrophils are activated during local tissue damage leading to degranulation and the subsequent recruitment of other cell types.

Accessory cells

Accessory cells work alongside lymphocytes in order to initiate an immune response. They include granulocytes and antigen-presenting cells such as dendritic cells.

Eosinophils, basophils and mast cells have a granular morphology and release various mediators upon activation. A key constituent of eosinophil granules is major basic protein, a cationic molecule capable of damaging pathogens directly. The release of IL-5 and eotaxin causes eosinophil recruitment to a site of inflammation. Eosinophils produce IL-2, IFN-γ, IL-4, IL-5 and IL-10. They also synthesise leukotrienes and prostaglandins, which are key mediators of the inflammatory response. Basophil granules contain inflammatory mediators such as histamine, IL-4 and leukotrienes. Histamine is also the major constituent of mast cell granules. Both basophils and mast cells have large amounts of IgE on their outer membrane and antigen engagement and cross-linking of these molecules is likely to cause activation.

Dendritic cells (DCs) are professional antigen-presenting cells and specialised DCs resident in the skin are known as Langerhans cells. Circulating DCs have an immature phenotype, which is associated with low-level MHC class II and costimulatory molecule expression, high endocytic activity and low T-cell activation potential. Upon encountering antigen, dendritic cells develop a mature phenotype which involves increased expression of MHC class II molecules, reduced endocytic capacity (Sallusto et al., 1995), expression of costimulatory molecules e.g CCR7, CCR1 and CCR5 (Sallusto et al., 1998) and secretion of IL-12 (Cella et al., 1996). Other signals that promote dendritic cell maturation include toll-like receptor signalling by bacterial components and pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and release of damage-associated molecular patterns (DAMPs) such as HMGB1, heat shock proteins and uric acid (Shi et al., 2003). The mature phenotype is associated with migration to the lymph. DCs also have a key role in

immune tolerance and are involved in negative selection of T-cells during thymic development (Brocker et al., 1997).

A number of drugs can influence both the maturation state and antigen-presenting capacity of dendritic cells. Amoxacillin induces the increased expression of maturation markers such as HLA-DR, CD80 and CD86 on dendritic cells from hypersensitive patients but not tolerant controls (Rodriguez-Pena et al., 2006). Sulfamethoxazole and sulfamethoxazole metabolites also induce enhanced expression of CD40 on dendritic cells isolated from healthy volunteers (Sanderson et al., 2007). This is not associated with cytotoxicity.

1.4.2 Antigen processing and presentation

The recognition of specific antigens by T-cells is a key element of adaptive immunity. As discussed previously, T-cells express TCRs that are specific for MHC-peptide complexes. In order for most antigens to be recognised they must be presented in the context of an MHC molecule.

Antigen-presenting cells process antigens by two distinct pathways (Figure 1.1). The first is directed at intracellular antigens. Intracellular antigens are often formed as a result of viral infection, whereby a virus enters the cell, replicates and hence viral peptides are released into the host cell cytosol. Proteins are degraded by the proteosome either in the cytosol or nucleus. Peptides are then translocated to the endoplasmic reticulum (ER) via a transporter associated with antigen processing (TAP)-dependent pathway. In the ER, the MHC heterodimer is formed, with the peptide fragment required for stability (Neefjes et al., 2011). This complex is then released and travels to the cell surface for antigen presentation. Uncoupled MHC/peptide molecules are returned to the cytosol for degradation (Hughes et al., 1997). MHC class I molecules present intracellularly derived peptides to $CD8^+$ T-cells.

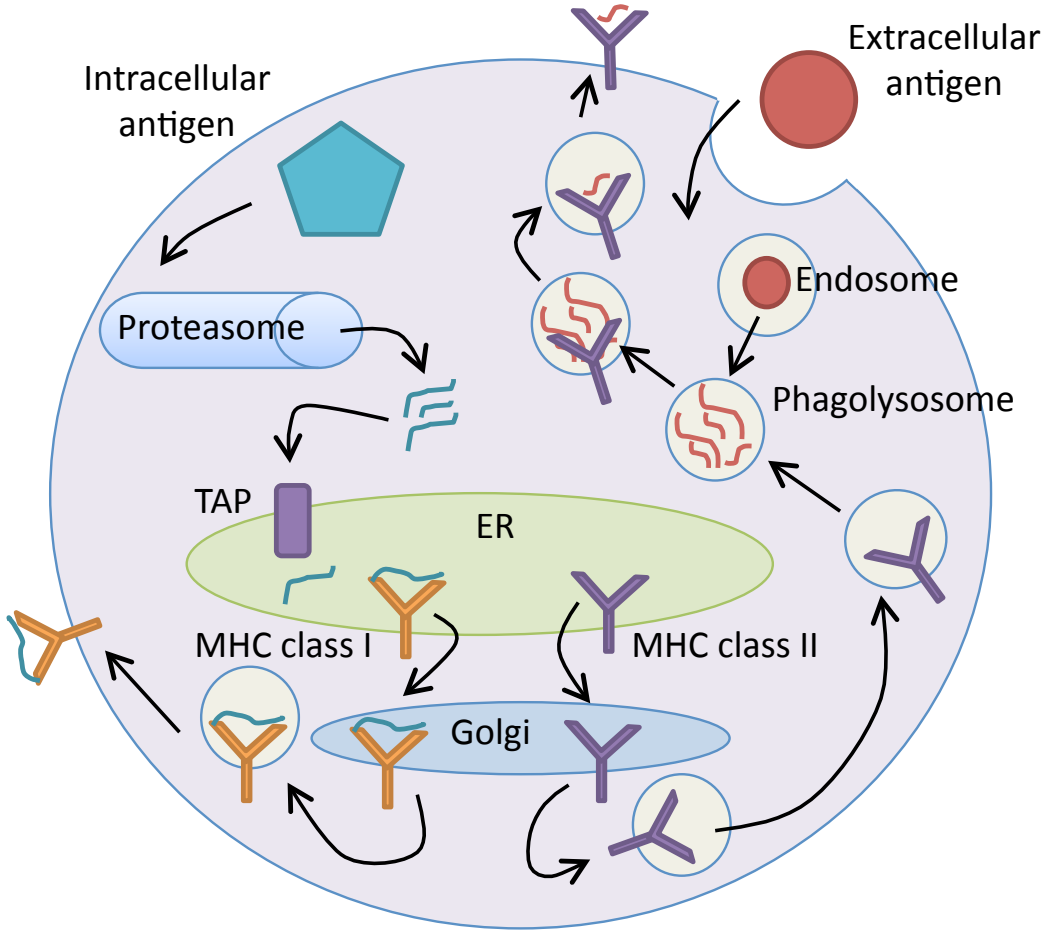


Figure 1. 1 Antigen processing and presentation. Intracellular antigens are degraded in the proteasome. Peptides are transported to the ER via TAP and associate with MHC class I molecules. The MHC-bound peptide is then trafficked to the golgi where it is loaded into a vesicle and transported to the cell surface. Extracellular antigens are taken up by phagocytosis and broken down by endosomal enzymes. MHC class II molecules are transported from the endoplasmic reticulum (ER) and golgi in vesicles. These vesicles fuse with the phagolysosome and this is where peptide loading occurs. The MHC bound peptide is then transported to the cell surface where the antigen is displayed. Adapted from (Neefjes et al., 2011).

Extracellular antigens are processed via a second pathway. Only specialised antigen-presenting cells such as dendritic cells and macrophages process antigens in this way. Pathogens are phagocytosed and contained within an endosomal vesicle where enzymes digest the pathogen into peptide fragments. MHC class II molecules are transported from the endoplasmic reticulum in membrane-enclosed vesicles, which then fuse with the endosome, allowing peptide loading to occur.

1.4.3 Intracellular signalling following TCR activation

Upon MHC-peptide engagement with the TCR, a complex signalling cascade is triggered eventually resulting in cellular proliferation, differentiation and/or cytokine/growth factor release. TCR-triggering i.e. the transfer of the signal across the cell membrane is a complex process, which is still not fully understood. Currently proposed models suggest either a conformational change, aggregation or segregation of the TCR-CD3 complex (Choudhuri et al., 2005). Much more is known about the signalling cascades following TCR triggering. Induction of tyrosine protein phosphorylation by the Src kinases Lck and Fyn is an early event in T-cell signalling (Nel, 2002). Phosphorylation of immunoreceptor tyrosine based activation motifs (ITAMs) within CD3 itself serves as a docking site for other proteins, in particular ZAP-70 (Smith-Garvin et al., 2009). In this way the TCR becomes a site for phosphorylation of a vast array of substrates initiating multiple signalling pathways that ultimately lead to T-cell activation. Targets of ZAP-70 are transmembrane adapter protein linker for the activation of T-cells (LAT) and SH2 domain-containing leukocyte phospho-

protein of 76kDa (SLP-76). This begins the formation of a complex of adapter and effector proteins positioned in order to ensure both proximity for subsequent enzymatic reactions and stability. The ultimate consequences of TCR activation include enzyme activation (e.g ERK, JNK, PKC), production of transcription factors (e.g NFAT, STAT3) and the release of signalling molecules such as Ca^{2+} and DAG.

1.5 T-cell activation by drugs

The activation of the immune system by small molecules such as drugs presents an interesting dilemma given that peptides are the traditional target of the immune response. A number of mechanisms by which small molecules can interact with immune receptors have been proposed and are outlined below. These mechanisms are not mutually exclusive.

1.5.1 Hapten hypothesis

Early studies dating back to the 1930s gave a first insight into the mechanisms underlying T-cell activation by small chemicals. Landsteiner and Jacobs were able to sensitise guinea pigs to low molecular weight, chemically reactive compounds such as dinitrochlorobenezene (DNCB) and hypothesised that protein conjugation may have an important role in the immune response observed (Landsteiner and Jacobs, 1935). Low molecular weight compounds such as drugs are unlikely to act as traditional antigens given their small size. It is suggested that immune recognition only occurs following the irreversible binding of a drug to host proteins. This is termed the hapten hypothesis. It

applies to directly protein reactive chemicals e.g dinitrochlorobenzene (DNCB) and drugs such as the β-lactam antibiotics. Most drugs however are not directly protein reactive and require bioactivation via drug metabolism in order to form reactive intermediates capable of binding covalently to protein (Castrejon et al., 2010, Elsheikh et al., 2011, Callan et al., 2009). This is termed the pro-hapten hypothesis. Drug/chemical modified proteins are then processed intracellularly where they are cleaved and associated with major histocompatability (MHC) molecules, before presentation and recognition by specific T-cell receptors (TCRs)(Figure 1.2). Direct binding to MHC molecules may also occur, or to peptides which are already associated with an MHC molecule. The source of antigen and location of any protein modifications (i.e. intracellular or extracellular) is thought to determine the site of processing although experimental confirmation of this has not been forthcoming. The class of MHC molecule that presents the peptide ultimately determines whether a $CD4^+$ or $CD8^+$ response is mounted. Advanced mass spectrometry techniques have been utilised to pinpoint precisely the amino acid residues that are modified by drugs such as sulfamethoxazole (Callan et al., 2009), piperacillin (Whitaker et al., 2011) and flucloxacillin (Jenkins et al., 2009) indicating that these can be highly specific.

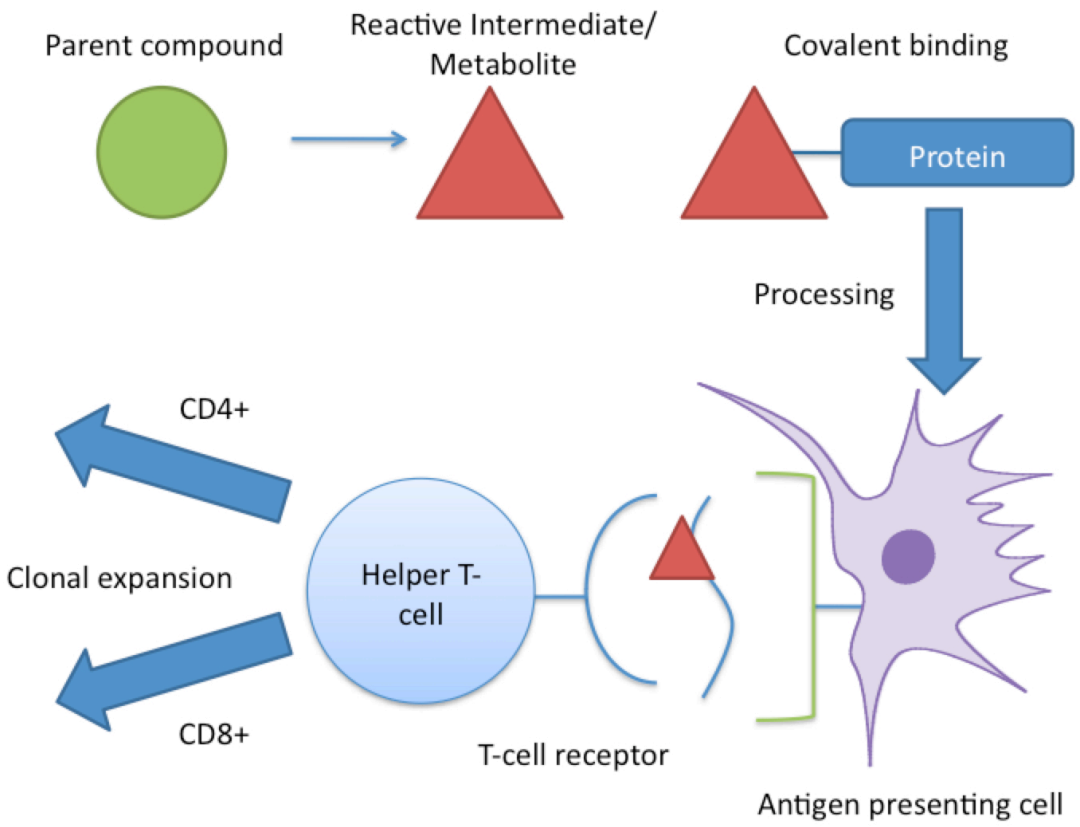


Figure 1.2 The hapten/pro-hapten hypothesis. Drug antigenicity occurs as a result of metabolism and subsequent covalent modification of host proteins. Modified proteins are then processed within the cell, cleaved into peptide fragments and associated with MHC molecules prior to translocation to the cell surface. It is the MHC/peptide complex that is recognised by the T-cell receptor. Antigens generated within the cell are processed via a proteasomal pathway, associate with MHC class I molecules, and elicit a $CD8^+$ T-cell response. Antigens generated outside of the cell are processed via an endocytic pathway, associate with MHC class II molecules and elicit a $CD4^+$ T-cell response (see Figure 1.1).

1.5.2 The pharmacological interaction (pi) concept

The ability of otherwise inert drugs to stimulate an immune response is somewhat at odds with the hapten hypothesis, and for this reason an alternative mechanism has been proposed. A direct, non-covalent association between the drug, TCR and MHC is suggested. This is known as the pharmacological interaction (pi) with immune receptors concept (Pichler, 2002). A direct, non-

covalent association is thought to occur between the drug molecule, and either the TCR or MHC molecule (Figure 1.3). This is thought to be a labile association, which is readily reversible. T-cells activated by a pi-mechanism respond rapidly to drug incubation as evidenced by Ca^{2+} influx, suggesting that processing pathways are bypassed (Zanni et al., 1998). As there is no *de novo* antigen formation it is hypothesised that stimulation via a pi-mechanism therefore represents stimulation of a pre-existing memory cell population (Adam et al., 2011).

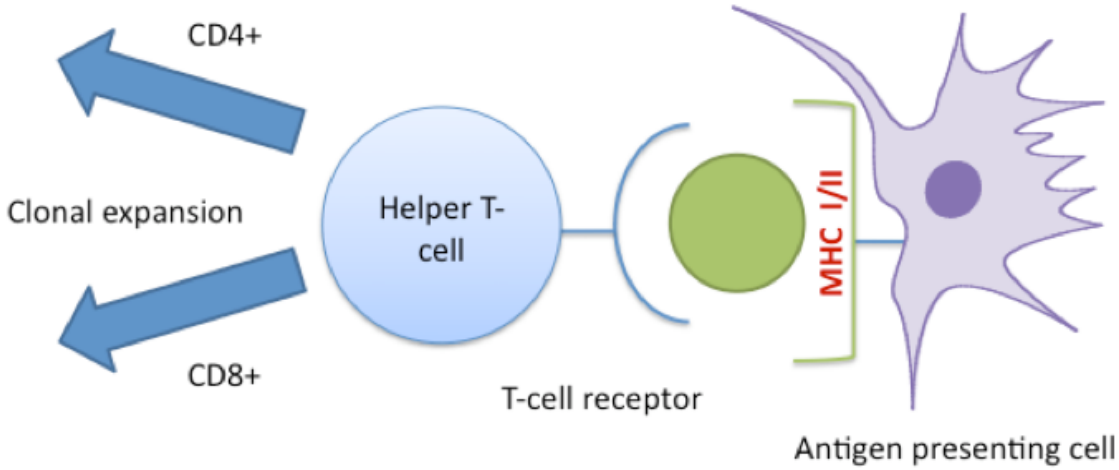


Figure 1.3. The p-i concept. A chemically inert drug is able to bind directly but non-covalently either to an MHC molecule or to the T-cell receptor, inducing an immune response. This may include hydrogen bonds, Van der Waals forces or hydrophobic interactions. A peptide may or may not be present.

1.5.3 Costimulation/danger hypothesis

The danger model was proposed by Matzinger in 1994. It states that immunity is not a simple case of self vs. non-self but rather it is the context in which an antigen is presented that determines whether an immune response is initiated (Matzinger, 1994). Immune activation is dependent on at least two signals.

Engagement of the TCR with an MHC-peptide complex represents signal one. This alone is not sufficient to stimulate an immune response. Signal two is derived from the interaction of various co-stimulatory molecules expressed on the antigen-presenting cell (APC) and the T-cell. In the absence of signal two, signal one leads to immune tolerance. The danger hypothesis relates to mechanisms by which signal 2 can be modulated. Both exogenous PAMPs (e.g LPS, peptidoglycan, viral RNA) and endogenous DAMPs released from damaged/dead cells (e.g. heat shock proteins, HMGB1) can contribute to danger signalling.

A number of drugs have been found to induce danger signalling:

- Sulfamethoxazole metabolites increase dendritic cell expression of CD40 (Sanderson et al., 2007).
- Abacavir induces heat shock protein redistribution in antigen-presenting cells (Martin et al., 2007).
- Amoxicillin drives dendritic cells towards a more mature phenotype (Rodriguez-Pena et al., 2006).

The potential of tissues to act as a source of danger signals or the local release of danger signals may explain why some reactions are confined or at least commence in certain tissues.

1.5.4 Terms used to describe small molecules

The following terms will be used throughout this thesis to describe the interactions of small molecules with the immune system:

- Immunogen

  A molecule that can stimulate a cellular or an immune response

- Antigen

  Any molecule that can bind specifically to an antibody or T-cell receptor

- Hapten

  A small molecule that only induces an immunological response when bound to a larger macromolecule

- Prohapten

  A small molecule, which following metabolism is able to bind to larger macromolecules and induce an immunological response.

- Co-stimulatory molecule

  A molecule that can stimulate or polarise an immune response to various antigens.

Any drug may exhibit several of these characteristics.

1.5.5 Experimental approach to study drug hypersensitivity reactions

A main focus of drug hypersensitivity research is the use of patient cells *ex vivo*. This is due to the importance of host factors in the development of immune-mediated reactions caused by drugs. A number of paradigm compounds illustrating different mechanisms of T-cell activation will be discussed.

Nitrohalobenzenes

Directly protein reactive compounds such as the contact sensitisers dinitrochlorobenzene (DNCB) and trinitrochlorobenzene (TNCB) have been utilised to explore the basis of the hapten hypothesis. These compounds belong to the larger class of nitrohalobenzenes, which all form the same dinitrophenol (DNP) or trinitrophenol (TNP) adduct, regardless of the substituted halogen (Figure 1.4A). Covalent binding has been observed at both cysteine and lysine residues (Maggs et al., 1986, Kitteringham et al., 1985) although it appears that protein modifications are selective, and dependent on the inherent reactivity of individual amino acids within a protein (Aleksic et al., 2007).

T-cell clones can be isolated from sensitised patients and are both $CD4^+$ and $CD8^+$ (Pickard et al., 2007), indicating that antigens are formed both intra- and extracellularly. The T-cell response is however processing-dependent and requires the presence of functional antigen-presenting cells. Confocal microscopy has revealed that extensive binding occurs throughout the cell within minutes of DNCB incubation (Pickard et al., 2007). Furthermore synthetic TNP-modified peptides are able to stimulate T-cells derived from mice originally sensitised to TNCB (Cavani et al., 1995). These peptides can be derived from many different sources (Kohler et al., 1995), though the positioning of the modification within the peptide can alter the strength of the response (Cavani et al., 1995). Importantly, the interaction of these haptens with MHC molecules has also been investigated, highlighting that these complexes can be recognised by T-cells (Weltzien et al., 1996). However, given the multiple targets of hapten formation it is difficult to suggest what the major

antigenic determinant will be *in vivo*. Given that all individuals exposed to nitrohalobenzenes can be sensitised, it is probably the case that multiple epitopes are capable of priming an immune response. MHC molecules will present high affinity peptides in certain individuals and low affinity peptides in others and this likely determines the relationship between exposure and the development of a clinical response.

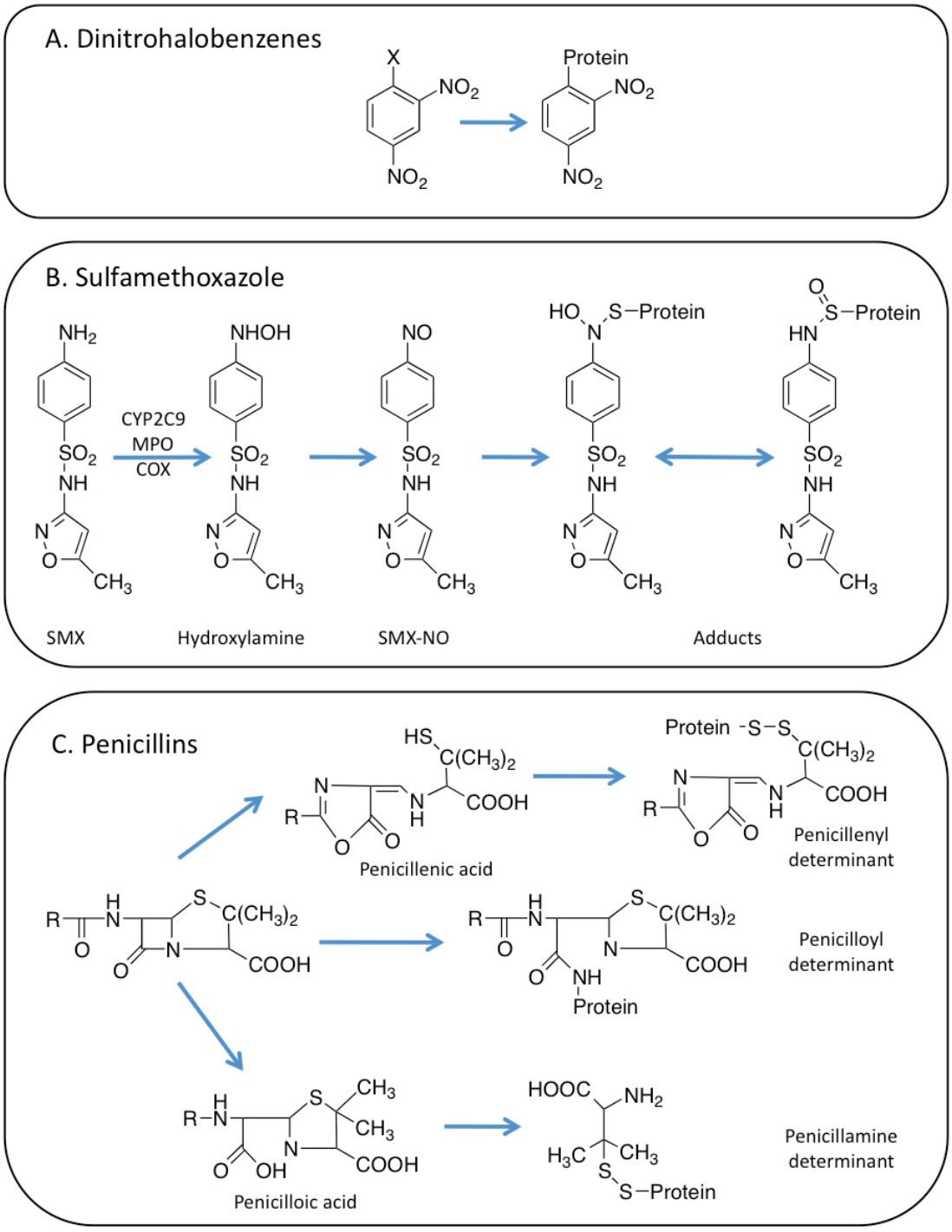


Figure 1.4 Antigen formation of model haptens. Dinitrohalobenzenes (A) all form the same dinitrophenol adduct regardless of the substituted halogen (X). A number of different penicillin adducts are formed following opening of the β-lactam ring (B). Sulfamethoxazole (C) is bioactivated to a nitroso metabolite via a hydroxylamine intermediate. Adapted from (Park et al., 2007).

Sulfamethoxazole

Hypersensitivity reactions to sulfamethoxazole occur in up to 8% of patients and are especially prevalent amongst cystic fibrosis and HIV patients (Lavergne et al., 2010, Carr and Cooper, 1995). The availability of synthetic protein-reactive metabolites, which are known to be formed *in vivo,* has aided the elucidation of the mechanisms of antigen generation (Figure 1.4B). Sulfamethoxazole undergoes CYP-mediated oxidation to form a hydroxylamine (SMX-NHOH) (Cribb et al., 1995). Reaction of SMX-NHOH with molecular oxygen then yields nitroso SMX (SMX-NO), which readily binds to proteins. Multiple mechanisms are thought to be involved in the T-cell response and T-cell clones isolated from hypersensitive patients reflect this. Distinct populations of T-cell clones can be generated that are either parent drug specific, metabolite-specific or cross-reactive (Castrejon et al., 2010). T-cell clones which are activated by the parent drug respond rapidly (Zanni et al., 1998) and in the absence of antigen processing (Schnyder et al., 2000) (Castrejon et al., 2010). Washing antigen-presenting cells pulsed with SMX for 1h abrogates the T-cell response (Castrejon et al., 2010). Metabolite-specific T-cell clones, in contrast, are activated rapidly by SMX-NO, but require longer incubation with SMX. This mirrors the time required for the formation of irreversible protein adducts *in vitro* (Elsheikh et al., 2011). T-cell responses to SMX-NO are blocked both by fixation of antigen-presenting cells and by addition of glutathione, indicating that the T-cell response to the metabolite is dependent on antigen processing and covalent binding (Castrejon et al., 2010). SMX and SMX-NO also induce co-stimulatory signalling, through the enhanced expression of CD40 (Sanderson et

al., 2007). Interestingly, in the case of the parent drug, this is prevented by the addition of an enzyme inhibitor (1-ABT).

Piperacillin

Studies involving the β-lactam antibiotic piperacillin illustrate how the combination of *in vitro* cell assays and advanced mass spectrometric techniques can be used to interrogate the mechanisms of antigen formation and presentation. The involvement of T-cells in penicillin allergy is well established. Drug-specific T-lymphocytes can be detected in the peripheral blood of hypersensitive patients via both LTT and cytokine secretion (Romano et al., 2004) (Rozieres et al., 2009) (El-Ghaiesh et al., 2012). T-cell clones can also be isolated, providing a platform through which the mechanisms of activation can be investigated.

There is long-standing evidence of β-lactams covalently binding to proteins and in particular, lysine residues (Levine and Ovary, 1961, Jenkins et al., 2009) (Padovan et al., 1997)(Figure 1.4C). This is due to the inherent reactivity of the β-lactam ring, which opens following nucleophilic attack. Stable protein adducts are subsequently formed. The penicilloyl product is the major antigenic determinant. Adduct formation is time-dependent, with extensive modifications to lysine residues on human serum albumin detectable following overnight incubation (El-Ghaiesh et al., 2012, Jenkins et al., 2009). Synthetic piperacillin-albumin conjugates can stimulate T-cell clones from hypersensitive patients in a concentration dependent manner (El-Ghaiesh et al., 2012). Furthermore, antigen-presenting cells incubated with piperacillin for 16 hours followed by

extensive washing are capable of stimulating T-cell clones to proliferate and secrete cytokines. Some clones will respond after pulsing for 4 hours, but pulsing for 1 hour will not stimulate clones (El-Ghaiesh et al., 2012), These time-points coincide with the *in vitro* formation of protein adducts.

A key finding of this work is that the synthetic adducts formed *in vitro* are also clinically relevant. Piperacillin-modified albumin could be detected in all of the patients undergoing therapy, irrespective of whether they had experienced a reaction or not (Whitaker et al., 2011). Protein modification occurred specifically at lysine residues as expected following *in vitro* incubation of albumin with the drug. Given that these protein conjugates were detected in all patients it seems clear that additional host factors must determine the susceptibility of an individual to DHRs.

1.6 Drug Metabolism

As stated previously, the formation of chemically reactive metabolites can result in the haptenation of proteins and subsequent immune recognition. In order to fully understand the mechanisms underlying T-cell activation by drugs it is therefore important to investigate drug metabolism alongside and in the context of cellular responses. This can be achieved through characterisation of the metabolising capacity of the different cell types utilised in *in vitro* assays and determination of the cellular fate of drugs and/or their metabolites. The basic principles of drug metabolism and the sources of chemically reactive metabolites will therefore be discussed here.

In order for a drug to reach intracellular compartments and its site of action, it must be lipophillic. However, lipophillic species are not readily eliminated. Drug metabolism is required to assist the effective excretion of drugs from the body and to prevent unwanted accumulation. Enzyme catalysed reactions convert the drug into a more water-soluble form, which can then be safely excreted in the urine. The liver is the main site of drug metabolism reactions although almost all cells are metabolically active.

1.6.1 Phase I drug metabolism reactions

Phase I drug metabolism reactions are catabolic and introduce or reveal functional groups such as hydroxyl, amino or thiol groups for subsequent conjugation in phase II. This is a process often known as 'functionalisation'.

The most common phase I reaction is oxidation. Reduction and hydrolysis reactions may also occur but are less frequent. If the product of phase I metabolism is sufficiently polar it may be excreted at this point.

Oxidation

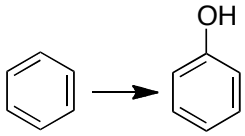
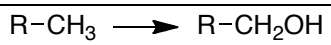
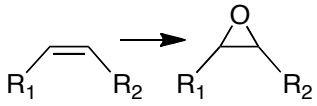
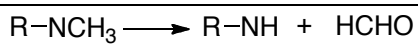
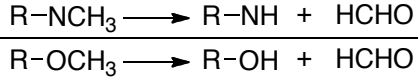
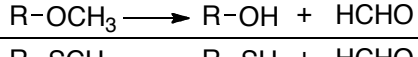
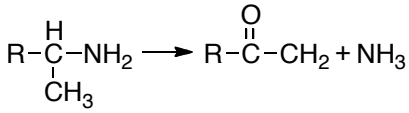
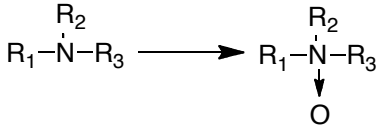
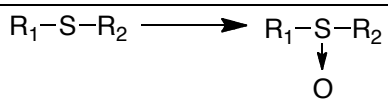
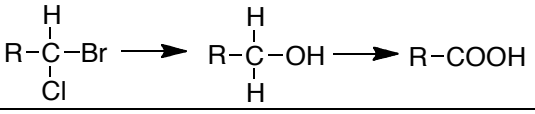
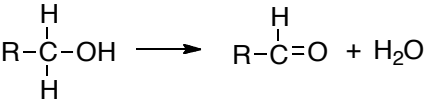
Cytochrome P450 enzymes (CYPs) are a superfamily of haem containing proteins which play a major role in oxidative phase I metabolism. There are dozens of CYP isoforms which are grouped into families i.e. CYP1, CYP2 etc. and subfamilies e.g CYP1A, CYP1B etc. based on their homology and sequence similarity (Nebert et al., 1987). Substrate specificity varies between isoforms though may be shared by subfamilies. Molecular oxygen, NADPH cytochrome P450 reductase and NADPH are required for CYP-mediated metabolism. A

summary of important phase I oxidation reactions catalysed by CYPs is shown in Table 1.4.

Key to the activity of CYPs is the central iron atom. Substrates bind to the ferric ($Fe3^{+}$) iron inducing a conformational change in the active site. Transfer of an electron from NADPH (via NADPH reductase) reduces ferric iron to its ferrous (Fe^{2+}) state. Ferrous iron then binds and reduces molecular oxygen following the transfer of a second electron. This forms a short-lived negatively charged peroxo group, which is rapidly protonated, resulting in the loss of an oxygen atom as water. The remaining oxygen is then inserted into the substrate, the product is released and the iron atom returns to its resting ferric state. The generalised formula of this reaction is shown below:

$$NADPH + 0_2 + RH + H^{+} \rightarrow NADP^{+} + ROH + H_2O$$

Table 1.4 Examples of phase I oxidation reactions catalysed by cytochrome P450 enzymes.

Reaction	General scheme	Substrate
Aromatic hydroxylation		Lignocaine
Aliphatic hydroxylation		Pentobarbitone
Epoxidation		Carbamazepine
N-Dealkylation		Diazepam
O-Dealkylation		Codeine
S-Dealkylation		6-Methylthiopurine
Oxidative deamination		Amphetamine
N-Oxidation		3-Methylpyridine
S-Oxidation		Chlorpromazine
Dehalogenation		Halothane
Alcohol oxidation		Ethanol

Compiled from (Gibson and Skett, 2001)

Though liver is the main site of drug metabolism some CYPs are specifically expressed in other organs. These include CYP2J2 which is heart-specific and CYP4A11 which is kidney-specific, though these isoforms are not known to have any significant contribution to drug metabolism. Lymphocytes, dendritic cells and keratinocytes express some CYP isoforms, in particular from the CYP1 superfamily (Sanderson et al., 2006). The presence of these enzymes is important to consider when discussing chemically reactive metabolites that

might only need to be formed in low levels in target organs to generate antigenic determinants that activate an immune response (see 1.6.4).

Other enzymes involved in phase I oxidative reactions include alcohol and aldehyde dehydrogenases, aromatases, amine oxidases and xanthine oxidase.

Reduction and hydrolysis

Reduction reactions can occur at nitro, keto or sulfoxide groups. Some reactions may involve CYP enzymes however molecular oxygen is not required. Hydrolysis reactions often occur in the plasma. They may be catalysed by tissue esterases or in some circumstances occur spontaneously. This is the case with acetylsalicylic acid (aspirin). Groups particularly susceptible to hydrolysis reactions include esters, which are hydrolysed to an acid and alcohol.

1.6.2 Phase II drug metabolism reactions

Phase II reactions are anabolic reactions. They often occur subsequent to a prior phase I reaction and result in a more polar, hydrophilic species, which can be effectively excreted in the bile or urine. As phase II reactions are conjugation reactions they generally require the formation of an energy-rich donor molecule. The most significant phase II reaction is glucuronidation which is catalysed by UDP glucuronyl transferase (UGT) located in the ER. Glucuronic acid must first be activated via conjugation with uridine diphosphate. This is then attached to the substrate. This is a major route of metabolism for carboxylic acid containing drugs, which subsequently form acyl glucuronides (Miners and Mackenzie, 1991). Some acyl glucuronides have been implicated in

drug toxicity due to their ability to bind covalently to protein *in vitro,* however whether these are the causal chemicals *in vivo* remains uncertain (Regan et al., 2010).

Some phase I reactions will result in the formation of potentially damaging electrophilic species. Reduced glutathione (GSH) is an important protective compound which is involved in the detoxification of these chemicals (Forman et al., 2009). It is a nucleophilic tripeptide, consisting of glycine, cysteine and glutamate residues. The cysteine residue contains a sulfhydryl group (-SH), which in its reduced state is able to donate an electron to electrophilic species. This reaction is catalysed by glutathione-S-transferase. Though glutathione conjugates may be directly excreted in the urine further metabolism may be required in order to yield a more hydrophilic product

1.6.3 Bioactivation and chemically reactive metabolites

As alluded to previously, the metabolism of some chemical compounds can result in the formation of chemically reactive metabolites (CRMs). These may have direct toxic effects through binding to specific 'critical' proteins e.g enzymes or transporters (Park et al., 2005a).

The two main types of CRM are electrophiles and free radicals (Williams et al., 2002) Electrophiles lack electrons and hence react with electron-donating nucleophiles. Both electrophiles and nucleophiles can be 'hard' or 'soft' and this determines the kinds of reactions they will be involved in (Jacobs, 1997). Soft electrophiles have a low energy unoccupied molecular orbital and are often uncharged. Targets of soft electrophiles include thiol groups found in cysteine

residues. Hard electrophiles have a high charge densitiy. Hard electrophiles react with hard nucleophiles such as basic groups in DNA and lysine residues. Metabolites with unpaired electrons are known as free radicals. Free radicals do not usually form covalent bonds, but rather abstract hydrogen molecules (Williams et al., 2002). This can lead to chain reactions and lipid peroxidation.

The ability of a compound to form CRMs is investigated during drug development and may influence whether a candidate is continued through to clinical trials (Park et al., 2011). These studies can be performed via two main methods. Covalent binding experiments can be performed with radiolabelled compound either *in vitro* or *in vivo* (Park et al., 2011). Adducts, in particular thioether or glutathione conjugates can also be detected via mass spectrometry. Incubations are performed *in vitro* with either microsomes or whole cell/tissue preparations or analysis of serum or urine following *in vivo* dosing can be achieved. Protein binding is however not always predictive of ADR risk. Indeed the formation of glutathione conjugates is indicative of bioinactivation, hence their detection suggests the efficient detoxification of CRMs *in vivo*.

1.6.4 Local production of chemically reactive metabolites

Though the liver is the main site of drug metabolism most cells in the body can be metabolically active. The local formation of chemically reactive metabolites may explain the organ-specific toxicity of some drugs and chemicals (Sanderson et al., 2006). A significant number of drug metabolising enzymes are expressed in skin and it is thought to be the site of bioactivation for many chemicals associated with contact dermatitis (Merk et al., 2007, Bergstrom et al., 2007, Ott

et al., 2009). Keratinocytes in particular are known to be metabolically active and express high levels of functional CYP enzymes (Baron et al., 2001). Other skin resident cells such as fibroblasts and melanocytes also express CYP mRNA (Saeki et al., 2002). Interestingly, a number of enzymes have been identified in skin which are not present at any significant level in the liver (Du et al., 2004). This may suggest that skin cells perform different biotransformations to liver cells and hence may be exposed to different metabolites, whether chemically reactive or otherwise.

The drug metabolising capacity of immune cells is less well investigated. Various cell types, including dendritic cells and lymphocytes, have been shown to express CYP enzymes at the mRNA level (Krovat et al., 2000) (Sieben et al., 1999) and CYP2B6 and 3A4 can be detected at the protein level in PBMCs (Liptrott et al., 2008). Drug-modified adducts can also be generated *in vitro* following incubation of SMX with antigen-presenting cells (Elsheikh et al., 2010). As yet there is no direct evidence that lymphocytes express functional metabolic enzymes or are capable of forming chemically reactive drug metabolites

1.7 The major histocompatability complex

Individual variation is a key aspect of drug hypersensitivity research. Polymorphisms in drug metabolising enzymes can impact upon the susceptibility of certain individuals to experiencing an adverse reaction. Recent work however also suggests a role for genes within the MHC region.

1.7.1 Location and variation

The MHC region comprises a cluster of genes spanning around 3.4Mb on the short arm of chromosome 6. Genes in this region have a key role in both adaptive and innate immunity. Strong linkage disquilibrium extends across the entire region such that groups of alleles may be inherited as haplotypes (Daly et al., 2001). The MHC region is the most polymorphic region in the human genome. Traditionally, this high level of polymorphism in this region has been attributed to the increased viral protection offered by heterozygosity (heterozygote advantage) (Doherty and Zinkernagel, 1975). Classical MHC genes encode a diverse array of cell surface glycoproteins involved in the presentation of antigens to T-cells (see 1.4.2). In humans, the MHC class I and II genes are specifically known as human leukocyte antigen (HLA) class I and II genes.

1.7.2 MHC Class I

MHC class I molecules are expressed by almost all nucleated cells. They include the HLA-A, HLA-B and HLA-C loci. Class I molecules consist of one gene

encoding a polymorphic light chain (α) and share a common β-chain (β_2m) the gene for which is not located in the MHC region (Figure 1.5). β_2m is not membrane bound but plays a key role in transporting newly synthesised MHC molecules to the cell surface. The peptide-binding groove accommodates peptides of between 9 and 11 amino acids in length and is restricted at both ends. Hydrogen bonds and Van der Waals forces secure the peptide in place.

1.7.3 MHC Class II

In contrast to the class I molecules, class II molecules are only expressed by specialised antigen-presenting cells such as dendritic cells, macrophages and B-cells. They are encoded by the HLA-DR, HLA-DQ and HLA-DP loci. The genes for both the α and β chains are located within the MHC region. Longer peptides can be accommodated in the peptide-binding groove of class II molecules as they are open at both ends.

1.7.4 MHC Class III

In addition to the genes encoding antigen-presenting MHC class I and II molecules, the MHC region is also the site of many other genes which play an important role in the immune response. These include loci encoding genes relating to the complement cascade, inflammatory mediators (e.g leukotrienes), cytokines (e.g TNF -α) and heat shock proteins (e.g Hsp70).

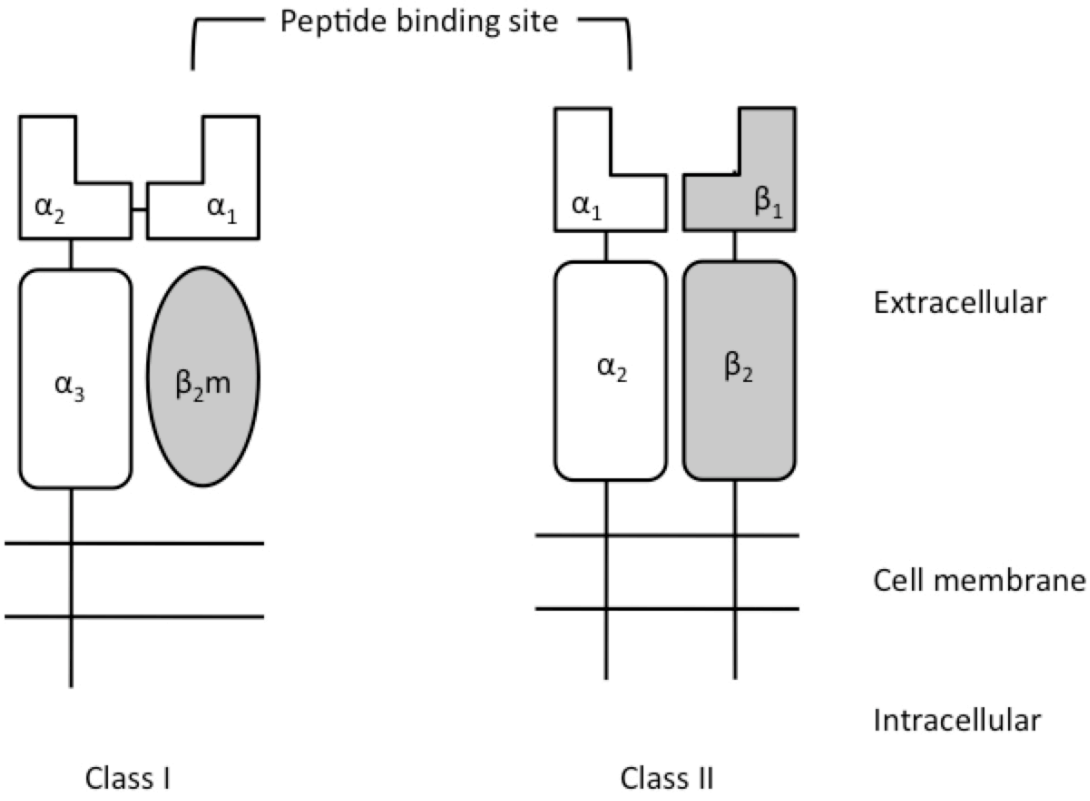


Figure 1.5 Structure of class I and class II major histocompatability molecules.

1.7.5 HLA nomenclature

The vast diversity of HLA alleles could not have been anticipated when they were first discovered, for this reason many changes have been made to the way in which they are named. Early work in the field was based on the observation that serum taken from patients that had received multiple blood transfusions caused the *in vitro* aggregation of platelets (Dausset et al., 1952). This was originally attributed to the presence of an antibody directed against the platelets. HLA-A and HLA-B antigens were identified shortly afterwards (Dausset, 1958). The HLA-C locus was not discovered until 1970 (Thorsby et al., 1970). The first class II alleles were identified eight years later (Albert et al., 1978).

Initially naming was assigned based on serological typing. This was performed using the microlymphocytotoxicity assay of Terasaki *et al* (1964). T and B-lymphocytes were isolated from the patient and exposed to a selection of antisera containing antibodies to individual HLA molecules. Antibody reactivity was used to identify the antigens expressed at the cell surface. Gene sequencing however, now means that differences in single amino acids that could not be identified by serologic techniques are now detectable. This necessitated a new method of naming alleles and it is this system that is still in use today. Figure 1.6 details the current system of naming HLA alleles. The letter immediately succeeding HLA e.g A, B, C etc. identifies the locus. The asterix immediately following signifies that the allele has been assigned based on molecular typing techniques. This is followed by a series of numbers. The first two digits

represent the type. In the majority of cases this matches the type that would be assigned by serology. The next two numbers identify the sub-type. These numbers are designated sequentially, as new alleles are discovered and signify differences in the amino acid sequence. The fifth and sixth digits are used to acknowledge polymorphisms that do not affect the peptide sequence. Digits seven and eight can also be used to identify polymorphisms in the non-coding region. Colons are now used to separate each of these numbers so that more than 99 variants can be accommodated in each sub-type i.e. variants are no longer confined to two digits (Tait, 2011). Letters are also included at the end of the series of numbers in order to give information about the expression of the allele, these include:

- N = null allele i.e. not expressed at the cell surface
- L = low expression at cell membrane
- S = expressed as a soluble product
- C = expressed in the cytosol but not the cell membrane
- A = aberrant expression
- Q = questionable expression, mutation known to affect expression

Meetings are held on a regular basis in order to discuss how to deal with the growing diversity of HLA alleles.

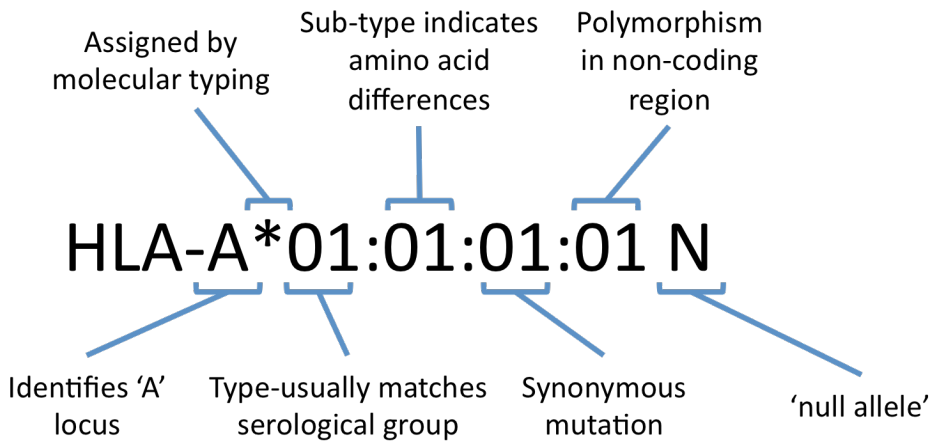


Figure 1.6 HLA allele nomenclature

1.7.6 HLA associations in adverse drug reactions

Recent genome-wide association studies suggest that one of the additional factors required for drug immunogenicity may be the genetic background of the patient, in particular, the possession of specific human leukocyte antigen (HLA) alleles. A first clue that genetic factors may be involved in the development of drug hypersensitivity reactions was a case of carbamazepine-induced mucocutaneous syndrome observed in monozygotic twins (Edwards et al., 1999). An association between HLA-B*57:01 and abacavir hypersensitivity syndrome was reported in 2002 (Mallal et al., 2002, Hetherington et al., 2002) and since then a number of genetic markers have emerged for both skin reactions (e.g carbamazepine (Chung et al., 2004, McCormack et al., 2011), allopurinol (Hung et al., 2005) and nevirapine (Martin et al., 2005)) and liver reactions (e.g lapatinib (Spraggs et al., 2011), lumiracoxib (Singer et al., 2010), ximelagatran (Kindmark et al., 2008) and flucloxacillin (Daly et al., 2009)) (Table 1.5). Associations are not confined to any particular clinical manifestation or any class of compound. However, the associations described

for liver reactions have to date been weaker than those observed for skin reactions. To further complicate the issue some associations can be confined to a particular ethnic group (Lonjou et al., 2008, McCormack et al., 2011) although this may reflect the different frequency of risk alleles in different populations.

The strength of a genetic association can be determined by calculation of an odds ratio (OR). This value compares the frequency of the risk allele in affected and unaffected groups (i.e. patients that have experienced an adverse reaction and those that have not) and can be calculated by generating a 2 x 2 table (Figure 1.7). It gives an indication of effect size.

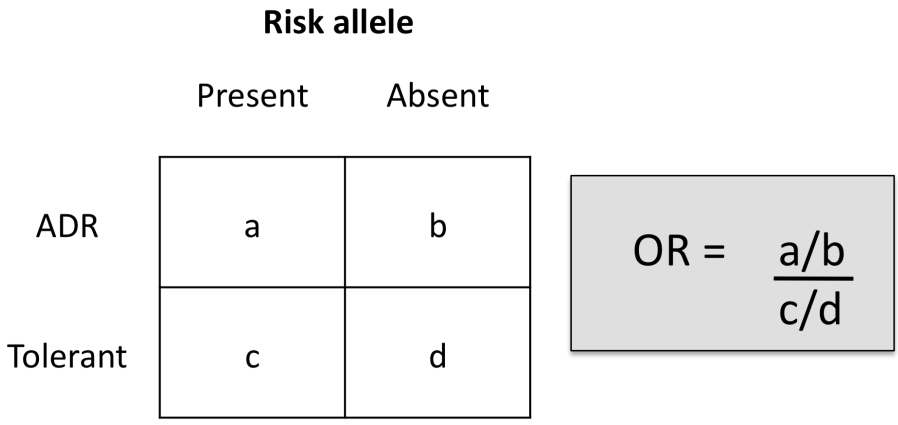


Figure 1.7 Formula for the calculation of odds ratios relating to the strength of associations of specific HLA alleles with adverse drug reactions.

Table 1.5 Summary of HLA-associated adverse drug reactions

Drug	HLA allele	Reaction (Ethnicity)	Odds ratio (95% CI)	Reference
Drug-induced hypersensitivity reactions				
Abacavir	B*57:01	Hypersensitivity (all)	117 (29-481)	(Mallal et al., 2002)
Carbamazepine	B*15:02	SJS/TEN (Han Chinese)	2504 (126-49,522)	(Chung et al., 2004)
	A*31:01	All phenotypes (Caucasians)	12.12 (4.03-20.65)	(McCormack et al., 2011)
Allopurinol	B*58:01	SJS (Han Chinese) (Caucasian)	580.3 (34.4-9780.9) 80 (34-187)	(Hung et al., 2005, Lonjou et al., 2008)
Nevirapine	DRB1*01:01	Hypersensitivity (Caucasian)	4.8 (1.3-16.8)	(Martin et al., 2005)
Lamotrigine	B*38	SJS/TEN (Caucasian)	6.8 (2.6-18)	(Lonjou et al., 2008)
Drug-induced liver injury				
Flucloxacillin	B*57:01	DILI (Caucasian)	80.6 (22.8-284.9)	(Daly et al., 2009)
Ximelagatran	DRB1*07:01 DQA1*02:01	DILI (Caucasian)	4.4 (2.2-8.9) 4.4 (2.2-8.1)	(Kindmark et al., 2007)
Lumiracoxib	DRB1*15:01	DILI (Caucasian)	5.3 (3.0 to 9.3)	(Singer et al., 2010)
Co-amoxiclav	DRB1*15:01 A*02:01	DILI (Caucasian)	2.3 (1.0 to 5.3) 2.2 (1.6-3.2)	(Donaldson et al., 2010, Hautekeete et al., 1999, Lucena et al., 2011, O'Donohue et al., 2000)
Antituberculosis (isoniazid, rifampicin, pyrazinamide)	DQB1*02:01	DILI (Indian)	1.9 (1.0 to 3.9)	(Sharma et al., 2002)
Lapatinib	DQA1*02:01	DILI (Caucasian)	2.2 (1.1 to 5.7) 9.0(3.2 to 27.4)	(Spraggs et al., 2011)
Diclofenac	DRB1*13	DILI (Caucasian)	-	(Daly and Day, 2009)
Clometacin	B*08	DILI	-	(Pariente et al., 1989)
Ticlopidine	A*33:03	DILI (Japanese)	13.0 (4.4 to 38.6)	(Hirata et al., 2008)

CI= Confidence interval; SJS= Stevens-Johnson syndrome; TEN= Toxic epidermal necrolysis; DILI= Drug-induced liver injury

The utility of screening for these alleles prior to prescription is dependent on a number of considerations, including the positive and negative predictive values, prevalence of the allele and transferability across different populations (Phillips and Mallal, 2010). In a recent study of flucloxacillin induced liver injury, over 80% of patients carried the HLA-B*57:01 allele compared to 6% of the controls (Daly et al., 2009). However, despite this strong association only 1 in 500 to 1 in 1000 individuals with the risk allele are likely to develop DILI, meaning that were prospective screening to be introduced a high number of patients who would not develop a reaction would be denied treatment (Daly et al., 2009). It has been estimated that over 13,000 patients would need to be tested in order to prevent one case of flucloxacillin-induced liver injury, compared to just 13 patients in abacavir hypersensitivity (Phillips and Mallal, 2010). It would therefore seem that the allele is required for the toxicity to develop but that other factors are also involved. This may include the immune status of the individual, history of infection or other concurrent mediations. In this case HLA-typing may serve as more of a diagnostic rather than predictive marker.

Genetic screening prior to prescription is now recommended for both abacavir and carbamazepine (in patients of Asian ancestry) and has been successful in reducing the incidence of DHRs to these drugs (Phillips and Mallal, 2009) (Rauch et al., 2006). As described previously the physiological role of HLA molecules is to present peptides derived from pathogens at the cell surface to T-cells. The observed genetic associations may therefore represent a functional consequence of polymorphism within the peptide-binding groove. With this in

mind *in vitro* assays utilising cells from a known genetic background are required and have been developed in this thesis.

The drugs abacavir, carbamazepine, flucloxacillin and ximelagatran will be discussed further.

1.8 Abacavir

1.8.1 Pharmacology and mode of action

Abacavir is a nucleoside reverse transcriptase inhibitor (NRTI) commonly used as part of combination therapy to treat human immunodeficiency virus (HIV). It was originally developed as an analogue of carbovir to reduce the toxicity and poor solubility associated with this molecule (Daluge et al., 1997). Substitution of the 3' alcohol with a cyclopropylamine group resulted in comparable potency with greatly improved pharmacokinetics. In rats, oral bioavailability was increased dramatically (Daluge et al., 1997).

Abacavir is a guanine analogue and inhibits viral reverse transcriptase through competition and chain termination of the growing viral RNA. As with other NRTIs, abacavir is a prodrug requiring intracellular phosphorylation. Three sequential phosphorylation steps must occur, the first of which is catalysed by adenosine phosphotransferase (Faletto et al., 1997). It is the stereospecificity of this enzyme in particular which is thought to be responsible for the lack of pharmacological action of the alternative enantiomer of abacavir. Figure 1.8 summarises the anabolic metabolism of abacavir. It is ultimately carbovir triphosphate that inhibits viral reverse transcriptase (Faletto et al., 1997).

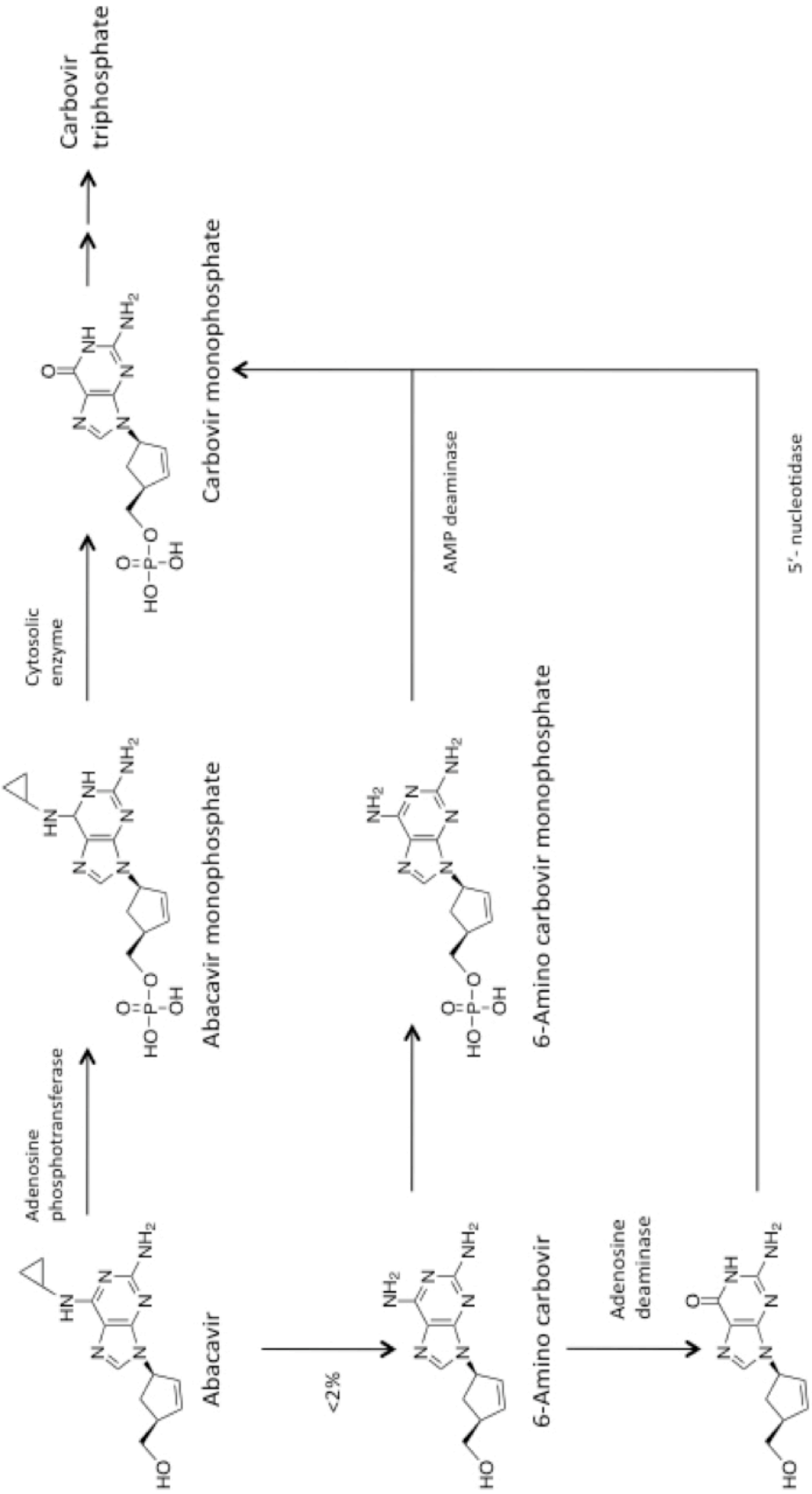


Figure 1.8 Intracellular phosphorylation of abacavir. Carbovir triphosphate is the active molecule, which inhibits viral reverse

1.8.2 Metabolism

In vivo, two major metabolites of abacavir are formed, a glucuronide and a carboxylic acid (McDowell et al., 1999). In urine these metabolites account for 36% and 30% of the administered dose respectively. The hepatic formation of these metabolites is catalysed by uridine diphosphate glucuronyl transferase (UGT) and alcohol dehydrogenase (ADH) (Walsh et al., 2002). The use of recombinant ADH isoforms has attributed this metabolism exclusively to class I isoforms, in particular ADH 1A1 and 1C2 (Walsh et al., 2002). The two-step oxidation process is thought to proceed via a reactive aldehyde intermediate (Walsh et al., 2002)(Figure 1.9). This intermediate has not been isolated due to its apparent stability however it can be trapped as an oxime when methoxylamine is added to enzyme incubations (Figure 1.10). Adduction reactions of synthetic abacavir aldehyde with valine residues have recently been characterised via mass spectrometry (Charneira et al., 2011). The mechanisms involved are thought to include both 1,4 addition (Figure 1.11) and Schiff base formation (Figure 1.10).

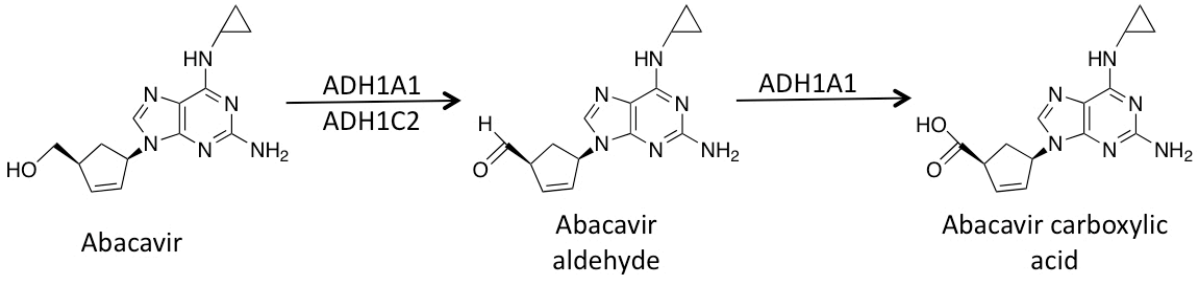


Figure 1.9 Abacavir oxidation reactions catalysed by alcohol dehydrogenase. *In vitro* and *in vivo* abacavir is oxidised to form carboxylic acid. This is thought to proceed via a reactive aldehyde metabolite, which can be trapped via the addition of methoxylamine.

1.8.3 Alcohol dehydrogenase

Alcohol dehydrogenase is a cytosolic enzyme most investigated for its role in the metabolism of ethanol. Interest in the enzymes has also recently grown in the field of cancer due to the carcinogenic properties of acetaldehyde, a by-product of ethanol metabolism (Jelski and Szmitkowski, 2008). There are six classes of ADH, which are defined based on their amino acid sequences. Enzymes in the same class generally share up to 60% homology (Jornvall and Hoog, 1995). It is a dimeric enzyme and contains a zinc atom. NAD is required as a co-factor.

The class I enzymes are the classical liver enzyme associated with the metabolism of ethanol. They are composed of α, β, γ sub-units which are encoded by the ADH1A, 1B and 1C genes respectively. Class I alcohol dehydrogenases are expressed primarily in the liver and adrenals (Edenberg, 2000). Moderate expression is found in the kidney and lung but they are not expressed in the heart or brain. A number of polymorphisms have been identified in both coding and non-coding regions which can influence the rate of ethanol detoxification, some of which are also associated with alcohol dependency (Edenberg, 2007). Class II ADH is a homodimer of the π sub unit. It is also expressed in the liver and is involved in ethanol metabolism. ADH class III is constitutively expressed throughout the body and is a homodimer of the χ subunit. It is encoded by the ADH5 gene (Edenberg, 2007). It is essentially a glutathione-dependent formaldehyde dehydrogenase (Koivusalo et al., 1989) and substrates include long chain alcohols. It has a low affinity for ethanol. ADH (classes I-III) is constitutively expressed in human skin (Cheung et al., 1999).

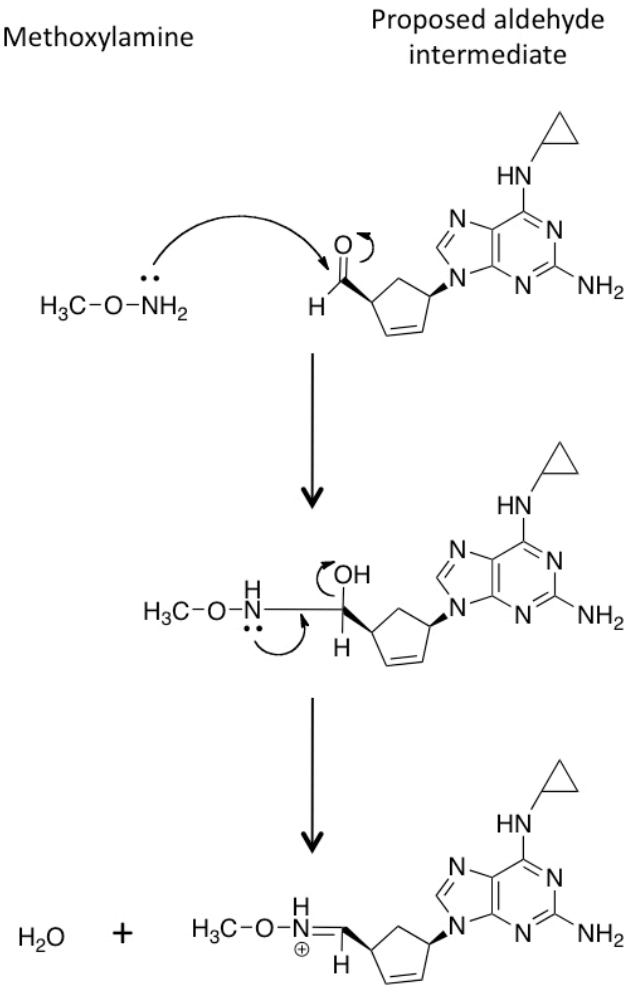


Figure 1.10 Trapping of abacavir aldehyde with methoxylamine via Schiff-base formation. Adapted from (Walsh et al., 2002).

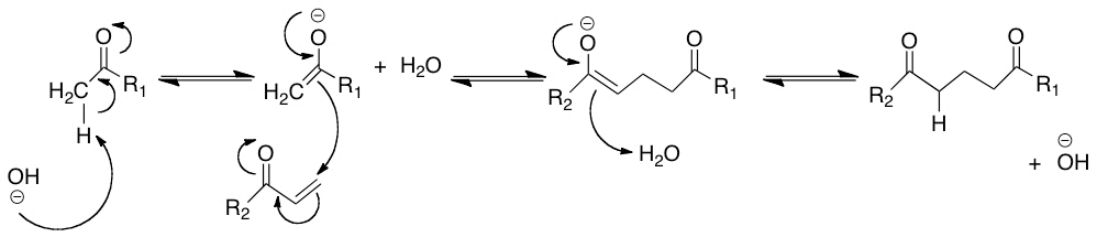


Figure 1.11 Mechanism of Michael addition of an enolate aldehyde or ketone to an α,β-unsaturated carbonyl compound on the β-carbon.

1.8.4 Hypersensitivity and HLA association

Hypersensitivity reactions to abacavir were observed early in clinical development (Saag et al., 1998). Symptoms were typical of HSS and included fever, nausea and skin rash within 6 weeks (median =11 days) of starting treatment (Hetherington et al., 2001). In 2002, two groups independently reported an association with HLA-B*57:01 and susceptibility to abacavir hypersensitivity (Hetherington et al., 2002, Mallal et al., 2002). Following a large-scale double-blind randomised prospective study, the effectiveness of genetic screening for the risk allele prior to prescription was established (Mallal et al., 2008) and has since been introduced on a global scale, effectively reducing the incidence of the reaction (Phillips and Mallal, 2009).

Epicutaneous patch testing, whereby the drug is applied to a small area of skin has improved the identification of true abacavir hypersensitivity (Phillips et al., 2002) and has also improved the predictivity of HLA-B*57:01 testing (Mallal et al., 2008). This is particularly important given that in some instances patients were diagnosed with HSS despite the fact that they were not receiving the drug (DeJesus et al., 2004, Gulick et al., 2006). Over estimation of the clinical problem may also be the reason for concerns having been raised regarding the transferability of the genetic marker across ethnicities (Hughes et al., 2004).

The immune basis for the adverse event is well established. Abacavir stimulates the specific release of pro-inflammatory cytokines, including interferon-γ (Almeida et al., 2008) and TNF-α (Martin et al., 2004) from peripheral blood mononuclear cells (PBMC) of hypersensitive patients *ex vivo*. Skin biopsies from

inflamed skin of hypersensitive patients show a marked infiltration of $CD8^+$ T-cells, suggesting that tissue damage develops as a result of cytotoxic T-cell activity (Phillips et al., 2002). The activation of antigen-presenting cells through redistribution of Hsp70 following abacavir exposure has also lead to the suggestion that the drug may induce signalling pathways leading to effective drug antigen presentation in individuals with the HLA-B*57:01 allele (Martin et al., 2007).

Innovative studies by Chessman *et al.* (2008) recently defined the role of HLA-B*57:01 in the disease pathogenesis. Abacavir-specific T-cell responses were detected *in vitro* using lymphocytes from healthy volunteers expressing HLA-B*57:01, but not other closely related HLA alleles (Chessman et al., 2008). The fine specificity of the abacavir MHC interaction was mapped to the B*57:01 antigen-binding F pocket where a serine residue at position 116 was important for drug antigen presentation.

1.9 Other adverse drug reactions with HLA associations

1.9.1 Carbamazepine

Carbamazepine (Figure 1.12A) is an anticonvulsant used in the treatment of epileptic seizures. Its action is caused by the blockade of voltage-gated sodium channels, which causes a reduction in the propagation of action potentials. It can also be used in the management of nerve pain and bipolar disorder.

Carbamazepine is a substrate for CYP3A4 and can be oxidised at a number of positions. Chemically reactive metabolites have been identified including an arene oxide (Madden et al., 1996) and a quinone (Ju and Uetrecht, 1999) however they have yet to be directly implicated in cellular damage. The 10,11 epoxide (Figure 1.12B) is an active metabolite and is further metabolised via epoxide hydrolase prior to excretion. Interestingly carbamazepine is also an inducer of CYP enzymes, meaning that it increases its own clearance after repeated doses. This can also lead to unfavourable interactions with other drugs such as the oral contraceptive.

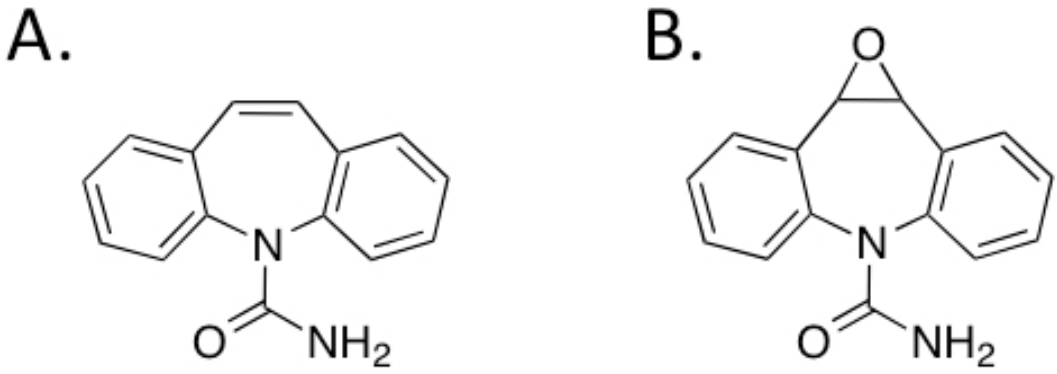


Figure 1.12 Chemical structures of carbamazepine (A) and its 10,11 epoxide metabolite (B).

A range of cutaneous adverse reactions have been associated with carbamazepine use, from the relatively mild MPE, increasing in severity to HSS and SJS/TEN. An immune pathogenesis has long been suggested based on the characterisitics of the reaction including time of onset and increased severity of the symptoms upon rechallenge (Leeder, 1998). Furthermore, PBMCs isolated from hypersensitive patients proliferate when exposed to carbamazepine *in vitro* (Naisbitt et al., 2003), indicating that T-cells are important in the recall response. The generation of T-cell clones has also provided a means to

investigate the phenotype and specificity of responding cells. Both $CD4^+$ and $CD8^+$ T-cell clones were identified, which also expressed the skin-homing receptors CTLA and CCR10 (Wu et al., 2007). Incubating the clones with analogues and metabolites of carbamazepine revealed a mixture of highly specific and cross-reactive T-cells (Wu et al., 2006).

In 2004, a case-control study performed in Taiwan found that the HLA-B*15:02 allele was present in all of the patients with SJS (44/44) but only 3% of carbamazepine-tolerant controls (3/101). The authors reported an odds ratio of 2504, a figure that remains one of the strongest HLA associations to be reported. A follow-up study however revealed that the genetic association only applied to SJS/TEN and not some of the milder reactions (Hung et al., 2006). Attempts to replicate the genetic association in other study populations have also met with mixed results. Studies performed with patients from a Han Chinese background have echoed the findings of the original study (Man et al., 2007, Locharernkul et al., 2008). In European studies however, the association with HLA-B*15:02 has not been repeated (Lonjou et al., 2008, Alfirevic et al., 2006). It has been suggested that this disparity could reflect both the increased frequency of SJS in Han Chinese populations compared to Caucasians (8 cases per million person years vs. 2-3 cases per million person years) (Chung et al., 2004) and the reduced frequency of the risk allele in Caucasians. The strength of the association in Han Chinese populations warrants prospective genetic screening in patients of this background and this is now reflected in drug labelling (Phillips and Mallal, 2010).

More recently a second genetic association has been reported from groups in both Japan and Europe suggesting that HLA-A*31:01 is a risk allele in these populations (McCormack et al., 2011, Ozeki et al., 2011). This association was considerably weaker than the original study but relevant to all forms of drug reaction and transferable across ethnicities. It is unclear what this suggests about the underlying pathogenesis of the reaction however, as it is unlikely that different routes of antigen formation and presentation are present in patients of different ethnicities.

1.9.2 Flucloxacillin

Flucloxacillin (Figure 1.13) is a β-lactam antibiotic used in the treatment of staphylococcal infections. It works by inhibiting the synthesis of peptidoglycan a crucial component of bacterial cell walls. Though generally well tolerated flucloxacillin use is associated with the appearance of cholestatic liver injury affecting the flow of bile, at a frequency of 8.5 in every 100 000 new users during the first 45 days of treatment (Russmann et al., 2005).

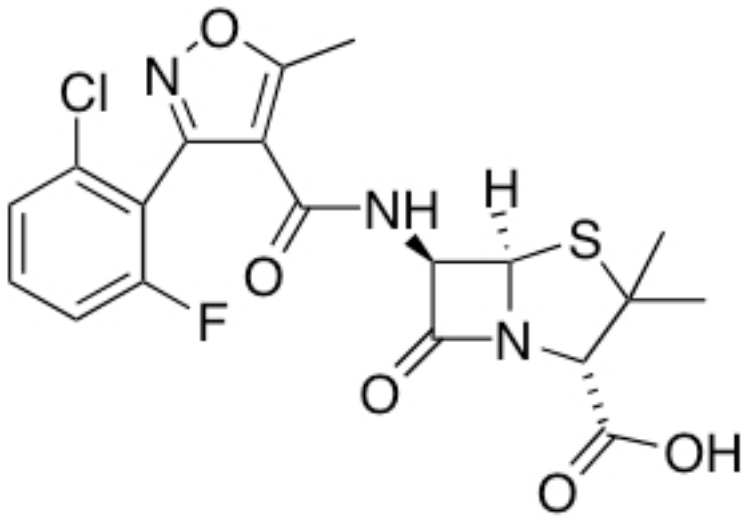


Figure 1.13 Chemical structure of flucloxacillin

A 5-hydroxymethyl metabolite formed via CYP3A4 metabolism has been identified as toxic to human biliary cells *in vitro* (Lakehal et al., 2001). When media from hepatocytes pretreated with flucloxacillin was added to cultures of biliary epithelial cells a significant increase in lactate dehydrogenase release was observed in 50% of preparations. Neither flucloxacillin nor the metabolite was directly toxic to hepatocytes. This does not however explain why only certain individuals develop liver injury.

In 2009 a GWAS study highlighted that 84% of patients (43/51) with flucloxacillin-induced liver injury carried the HLA-B*57:01 allele, compared to just 5% of the population controls (Daly et al., 2009). Interestingly the HLA allele (B*57:01) identified is also associated with abacavir hypersensitivity reactions. The association may therefore indicate a possible role for T-cells in the pathogenesis of the reaction. Indeed positive LTTs to flucloxacillin have previously been recorded (Maria and Victorino, 1997). The reasons why liver is targeted however are unclear although a role for drug metabolism could be hypothesised. Both flucloxacillin and the 5-hydroxymethyl metabolite covalently bind to HSA and at very similar positions within the protein (Jenkins et al., 2009). This has been shown both *in vitro* and also *in vivo*, where modified peptides could be detected in the plasma of all patients examined (8/8), this may therefore implicate hapten formation in the adverse reaction.

1.9.3 Ximelagatran

The novel, direct thrombin inhibitor ximelagatran was withdrawn from the market in 2006 following the observation of raised liver transaminases 3 x ULN

in 7.9% of patients taking part in long-term clinical trials (Lee et al., 2005). This was however generally asymptomatic and resolved in the vast majority of patients (96%) regardless of whether ximelagatran treatment was terminated.

Ximelagatran is a prodrug for the active molecule melagatran. Melagatran is a potent competitive inhibior of thrombin, preventing the polymerisation of fibrinogen, the final step in the clotting cascade. The molecule resembles a short peptide with the peptide bond able to fit in the arginine pocket of thrombin. Though melagatran was effective when dosed parentreally, oral dosing was not as effective. This is because melagatran contains both a strongly basic benzamidine group (pKa=11.5) which carries a positive charge at intestinal pH and a carboxylic acid group (pKa=2) which is ionised at physiological pH (Gudmundsson, 2007). Ximelagatran was therefore developed to shield these groups and displays improved gastrointestinal absorption (Gustafsson et al., 2001) It is absorbed rapidly following oral administration, has a rapid onset of action and shows little inter-individual variability (Eriksson et al., 2003). Ximelagatran can also inhibit thrombin but is much less potent.

The conversion of ximelagatran to melagaatran is catalysed by tissue esterases (Figure 1.14) and requires both a reduction and a hydrolysis reaction, which can occur in either order. Reduction of the hydroxyamidine yields ethyl-melagatran whilst hydrolysis of the ethyl ester forms OH-melagatran. Ximelagatran is not a substate for CYP enzymes (Bredberg et al., 2003)

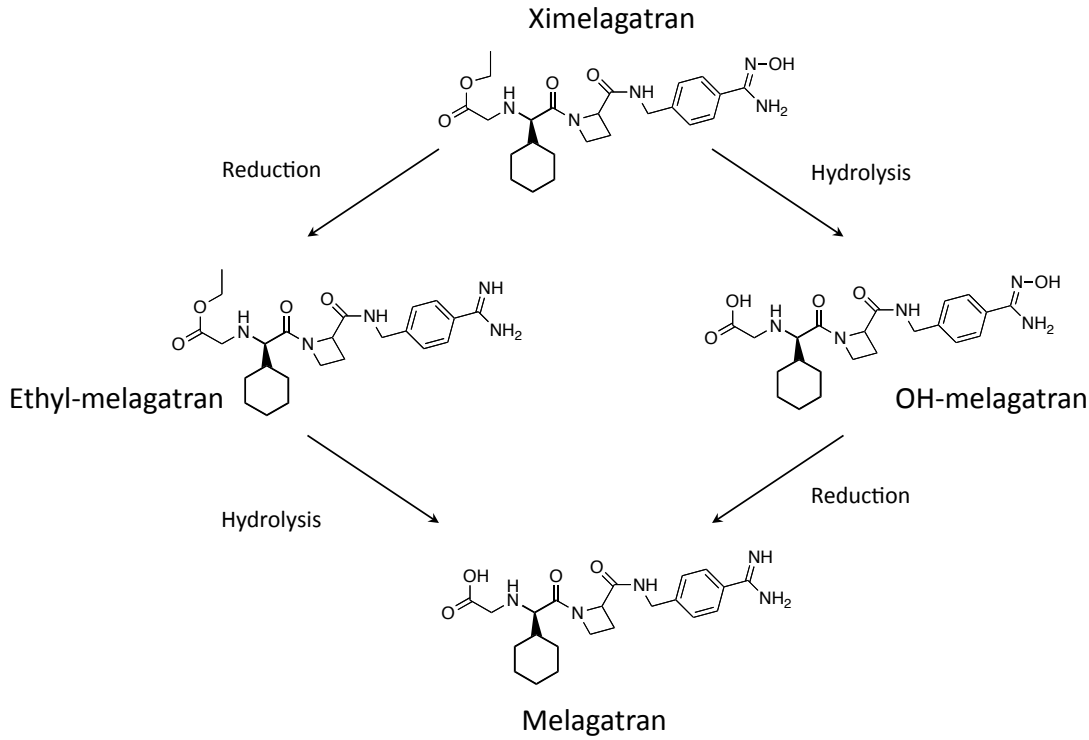


Figure1.14 Metabolism of ximelagatran to the active molecule melagatran

Studies performed in isolated hepatocytes and hepatoma cell lines have not detected any direct toxic effects of either ximelagatran or metabolites (Kenne et al., 2008, Edling et al., 2008). Kenne et al. (2008) used a barrage of *in vitro* assays including measurement of ATP, intracellular Ca^{2+}, mitochondrial function, formation of ROS and glutathione conjugates and only observed any loss of viability at 100-200μM ximelagatran. Though these studies were only performed on a short-term basis (<48h) the concentrations at which any loss in viability was observed greatly exceed those reached *in vivo* (mean plasma C_{max} = 0.3μM) (Wolzt et al., 2003).

Currently an immune pathogenesis is proposed. This is supported by positive lymphocyte transformation tests and patch tests in occupationally exposed workers, suggesting the involvement of circulating lymphocytes (Kindmark et al., 2008). A clear geographical distribution has also been observed, with a higher incidence of the reaction seen in Northern Europe (Kindmark et al., 2008). A genome wide association study performed among 74 cases and 130 controls highlighted HLA-DRB*07:01 and HLA-DQA*02:01 as the most strongly associated with an increase in ALTs ($p = 4 \times 10^{-5}$) (Kindmark et al., 2008). Further investigation into this relationship and how the drug interacts with these alleles to elicit toxicity in these individuals will provide insight into off-target toxicities and factors influencing individual susceptibility.

The structure of ximelagatran is such that it resembles a small peptide fragment, and this may be important in the development of an immune-mediated reaction (Uetrecht, 2008). The ability of the compound to bind to the thrombin active site may suggest that it is also able to bind to other proteins, such as MHC molecules. Interactions with protein, whether covalent or non-covalent in nature, are suspected to be important in the development of immune responses (Park et al., 2005b). The fact that other drugs marketed in this class e.g dabigatran do not cause immune-mediated reactions may suggest that this is not the only factor (Hankey and Eikelboom, 2011).

1.10 Aims

Recently observed HLA associations suggest a key role for specific cell surface antigens in the pathogenesis of immune-mediated adverse drug reactions. *In vitro* assays incorporating this genetic variation are required in order to account for allele-specific mechanisms. The aims of the work presented in this thesis were therefore to;

- Establish a cohort of HLA-typed healthy volunteers from which lymphocytes and DNA can be collected
- Develop *in vitro* assays to detect drug responsive T-cells from volunteers expressing HLA alleles associated with ADRs
- Characterise the phenotype and functionality of drug reactive T-cells
- Investigate the metabolism of abacavir in cellular assays

Chapter Two

Establishment of a HLA typed cohort to elucidate the cellular and chemical basis of adverse drug reactions

2.1 Introduction

Human leukocyte antigen (HLA) molecules are cell surface molecules that present peptides to T-cells. If these peptides are derived from an invading pathogen immune recognition will occur. Recent genome wide association studies have also suggested a key role in the development of drug hypersensitivity reactions. For both abacavir (Chessman et al., 2008) and carbamazepine (Ko et al., 2011) the associated HLA molecule has been suggested to have a direct role in the presentation of drug/drug-modified peptides to T-cells. In order to study the mechanisms of T-cell activation *in vitro,* cells bearing these molecules must therefore be used. A cohort of 400 healthy volunteers has been established in order to provide a source of genetically characterised lymphocytes.

HLA *Typing*

The involvement of specific HLA alleles in adverse drug reactions and in tissue rejection makes reliable HLA typing key to improving drug therapy and preventing graft vs. host reactions. There are several methods currently in use to identify patient genotype.

Serological HLA typing techniques

The first methods routinely used in HLA typing were based on the microlymphocytotoxicity assay of Terasaki *et al* (Terasaki and McClelland, 1964). T and B-lymphocytes were isolated from the patient and exposed to a selection of antisera containing antibodies to individual HLA molecules. Fixation

of complement and subsequent cytotoxicity were used as a measure of antibody reactivity and hence to identify the antigens expressed at the cell surface. Serological techniques can however be limited by the quality and availability of antisera for specific HLA alleles (Otten et al., 1995). Antibodies directed at HLA molecules generally only recognise differences at the surface of the molecule, making it difficult to characterise HLA molecules that only differ at a couple of residues, particularly when these are located in the peptide binding cleft (Shankarkumar et al., 2008) and as such only low resolution typing can be performed (Williams, 2001). This method is also compromised in situations where disease states affect the expression of antigens at the cell surface (Williams, 2001). Otten *et al.* (1995) found that discrepancies between serological and genotyping techniques were increased in patients compared with healthy volunteers.

Genotyping techniques have since been developed which can identify HLA type at the gene level, and can be performed on any source of genomic DNA (Susskind, 2007).

Sequence-specific oligonucleotide probe hybridization (SSOPH)

Primers complementary to the conserved 5' and 3' flanking regions of the locus of interest are designed. Amplified products are then incubated with a range of sequence-specific oligonucleotide probes, and an allele is called based on any resulting hybridization. A vast number of oligonucleotides are required due to the number of alleles that have currently been identified, and this can impact on the costs of the assay (Williams, 2001). These methods are not dependent on

surface expression of the antigen and the ability to discriminate single base changes between alleles means that a much greater degree of resolution is achieved. Additional oligonucleotides can be designed as new alleles are discovered.

Sequence-specific primer polymerase chain reaction (SSP-PCR)

Primers are designed to the polymorphic region of the allele, meaning that, in this instance an allele can be assigned when amplification occurs, as only an allele that is perfectly aligned with the primer will be amplified (Williams, 2001). These products can then be separated by electrophoresis to determine whether amplification occurs, or real time PCR methods can be employed (Williams, 2001).

Large amounts of DNA are required to fully characterize patient HLA type via this method, due to the number of reactions that must be performed and primers used (Susskind, 2007). However the implementation of whole genome amplification techniques has aided this method, so that a mouth swab can be sufficient for repeated typing experiments (Gillespie et al., 2000). SSP-PCR is useful in typing at a standard equivalent to serology (Williams, 2001). Both SSP-PCR and SSOPH-PCR can be performed in 4-5 hours (Susskind, 2007).

Direct DNA sequencing

PCR amplification of genomic DNA followed by sequencing of the amplified regions is currently the most comprehensive HLA typing technique, yielding high-resolution allelic discrimination (Shankarkumar et al., 2008).

Fluorescently labelled nucleotides are employed to determine the sequence of the amplified regions, base by base (Williams, 2001)(see 2.3.2).

However, interpretation of sequence-based typing results is more difficult than in other methods. Specialist software and a skilled operator are required in order to make allele calls (Sayer and Goodridge, 2007). Sayer *et al.* (2007) found both inter- and intra- laboratory variation exist in sequence-based techniques, and that this was largely due to operation rather than specific reagents or equipment. Direct sequencing is also a much more time consuming process, sometimes taking a number of days (Susskind, 2007). Genotyping techniques can give conflicting results when null alleles are present (Elsner and Blasczyk, 2004). These null alleles that are not present at the surface can still be detected by PCR techniques, a factor that may be important in transplantation (Elsner and Blasczyk, 2004).

Though undoubtedly useful, genotyping techniques can be expensive and time-consuming processes, requiring specialist equipment and complicated analysis. Following the characterization of linkage disequilibrium throughout the MHC region a number of tagging single nucleotide polymorphisms (tSNPs) have been identified (de Bakker et al., 2006). These tSNPs offer an alternative method of predicting HLA types, which can be performed in most laboratories without the need for specialised equipment.

The MHC region is organized into a series of distinct haplotype blocks (Daly et al., 2001). The arrangement of these blocks is such that particular SNPs are

inherited together at a high frequency. Those SNPs that are tightly linked can be said to be predictive of other SNPs located within the same haplotype. In this way probing of tagging SNPs can yield information relating to SNPs at distant sites (de Bakker et al., 2006).

The presence of HLA-B*57:01 strongly predicts the development of a hypersensitivity reaction to the commonly used antiretroviral drug abacavir (Mallal et al., 2002), so much so that prospective HLA typing has been adopted by many clinicians. A tagging SNP for this allele has been proposed which we have also sought to investigate here.

2.2 Aims

- Establish a biobank of lymphocytes from HLA-typed healthy volunteers.
- Investigate the use of the HCP5 SNP as a tagging SNP for HLA-B*57:01.

2.3 Methods

2.3.1 Volunteer recruitment and eligibility

Individuals responsible: Jane Evely and Giovanna Bretland (CDSS nurses)

Ethics for this study were obtained from the Liverpool Research Ethics Committee and all participants gave written informed consent. Advertisements were placed in local newspapers, on the University of Liverpool website and the Royal Liverpool University Hospital bulletin. Volunteers were eligible to participate in the study if aged between 18 and 60 years, healthy and willing to donate one or more blood samples. The following exclusion criteria were applied and volunteers were not recruited if:

- they had donated blood to the transfusion service in the last 4 months;
- they had any medical problems including asthma, diabetes, epilepsy or anaemia;
- they were on any medications;
- they had taken any recreational drugs in the past 6 weeks (including cannabis, speed, ecstacy, cocaine, LSD etc.).
- Women were excluded if pregnant.

400 healthy volunteers were recruited from the North West of England between August 2009 and April 2010. This sample size was based on the frequency of the HLA-DRB*07:01 allele, associated with ximelagatran-induced liver injury (Kindmark et al., 2008). Based on an allelic frequency of 0.165, a cohort of 400 volunteers was therefore expected to identify more than 110 heterozygotes and around 10 homozygotes.

Blood was taken at the Royal Liverpool and Broadgreen Hospital Clinical Research Facility. Confidentiality was maintained throughout the study by several mechanisms: firstly, only clinically trained personnel had access to participant's personal data which are stored in password-protected computer files; secondly, coding of DNA and cells used in functional assays were performed; thirdly, subjects were identified only by their assigned number. Participants were given the right to withdraw from the study at any time. In that case, any identifiable data or tissue would be anonymised and retained or otherwise disposed of if specified by the participant.

A total of 100ml of blood was collected for both DNA and peripheral blood mononuclear cell (PBMC) isolation. Careful consideration was paid to ensuring that the amount of blood collected would enable the intended experimental strategy without asking too much from volunteers. Further donations may however be requested at a later date in order to replenish cell stocks. All samples were processed within 4 hours of collection.

2.3.2 Extraction of genomic DNA and sequence-based HLA typing

Individuals responsible: Vivian Platt

Genomic DNA was extracted from whole blood using the automated Chemagic magnetic separation system (Chemagen, Baesweiler, Germany). Firstly, cells were lysed. Lysis buffer (7.5ml) and protease (20µl) were added to 3-5ml of whole blood and incubated for 5 minutes at room temperature. The Chemagic

magnetic separation module (Chemagen, Baesweiler, Germany) was then run on a lysis cycle for 30 minutes. Following lysis of the cells 19.5ml of binding buffer was added along with 0.5ml magnetic beads. The magnetic beads are able to bind DNA with a high affinity allowing the transfer of the beads from one buffer to another when the electromagnetic field is turned on. Resuspension of the nucleotides is achieved when the magnet is turned off through high-speed rotation of the rods allowing efficient washing. The DNA was washed in 10 ml of five separate wash buffers followed by 0.5 ml of two elution buffers as provided by Chemagen. DNA concentration was determined by measuring absorbance at 260nm (A_{260}) using the Nanodrop spectrophotometer (Labtech, East Sussex, UK). High-resolution sequence-based HLA typing was performed at the Histogenetics laboratory (Histogenetics New York, USA) at the following loci; HLA-A, B, C, DRB1 and DQB1. Samples were sent for HLA-typing in two batches of 200. Methods were as described in detail in Lucena et al. (2011) and are briefly summarised here. Sequencing was performed on the PCR products generated from exon 2 of HLA-A, B, C, DRB1 and DQB1. Exon 3 was additionally sequenced for the class I alleles. Sequencing was performed on both strands via cycle sequencing with BigDye V3.1 (Applied Biosystems, Warrington, UK). Analysis of the resulting data was performed using Histomatcher and HistoMagic software developed at Histogenetics (New York, USA). Alleles were assigned based on the latest version of the IMGT/HLA database (release date April 2008).

Long-term storage of DNA from all volunteers is provided by SmaRTStore, a DNA archive with integrated robotics (at -20˚C). Samples can be tracked and

monitored by means of the electronic laboratory management system, STARLIMS. A small amount of whole blood was also retained and stored at -80˚C should further DNA need to be extracted. Blood spots were also prepared for each volunteer.

2.3.3 Isolation and storage of volunteer lymphocytes

Individuals responsible: Catherine Bell and Klara Martinsson

Peripheral blood mononuclear cells (PBMCs) were isolated from 90ml of blood, which was collected into heparinised vacutainer tubes. Blood (25ml) was layered on top of lymphoprep (25ml) (Axis-shield, Dundee, UK) and the erythrocytes sedimented via density centrifugation (400g, 25min, room temperature). The buffy coat layer containing the PBMCs was then carefully aspirated with a pasteur pipette. PBMCs were washed twice in Hanks balanced salt solution (HBSS) to remove any remaining lymphoprep and resuspended in 10ml HBSS. A 10µl aliquot was then added to 40µl HBSS and 10µl of this diluted cell suspension was then added to 10µl trypan blue (0.2 % w/v). A 10µl aliquot of stained cells was then counted on a Neubauer haemacytometer (Sigma-Aldrich, Dorset, UK) under a Leica DME microscope (Leica Microsystems, Milton Keynes). Cell viability was assessed by trypan blue exclusion and calculated using the following equation: percentage viability = 100 x viable cells ÷ total cells. Viability was typically >95%.

PBMCs were resuspended in foetal bovine serum (Invitrogen, Paisley, UK) containing 10% DMSO (Sigma-Aldrich, Dorset, UK) at a density of 10^7 cells/ml and frozen at -150˚C. A tracking system exists in order to locate and record the

number of cells remaining from each volunteer. When required, samples will be thawed and viable cells counted.

2.3.4 Tagging SNP identification of HLA-B*57:01

In order to allow preliminary experiments on genotyped cells to be performed DNA from 23 volunteers was utilised in a PCR-based assay to investigate the use of the rs2395029 (HCP5) SNP as a specific marker of HLA-B*57:01. The HCP5 SNP is a T to G substitution located 100kB centromeric to the HLA-B locus resulting in a valine to glycine missense mutation.

PCR reactions were set up as follows; 20ng DNA was added to TaqMan genotyping master mix (Applied Biosystems, Warrington, UK) and the primers (Applied Biosystems, Warrington, UK) in a 96-well PCR plate. A negative control, containing no DNA was also included to rule out contamination of the MasterMix. Thermal cycling was performed by ABI Prism 7000 sequence detection system (Applied Biosystems, Warrington, UK) (10min at 95˚C followed by 40 cycles of 15s at 95˚C and 60s at 50˚C). At the end of the programme a post-read cycle was run in order to allow allelic discrimination. Accuracy was assessed by running 10% of the samples in duplicate. Following PCR amplification of the regions of interest, ABI prism software was used to discriminate between individual genotypes.

2.4 Results

2.4.1 Volunteer demographics

400 volunteers were recruited, however only 385 were able to donate sufficient blood to enable both DNA and lymphocyte isolation. Of these 400 volunteers 64% were female and 36% were male. The average age of volunteers was 29 years (±10 years range 18-60)(Figure 2.1). Given that a high number of university students were recruited there is a higher proportion of volunteers below the age of 30. Recruitment of volunteers began in August 2009 and was completed in April 2010.

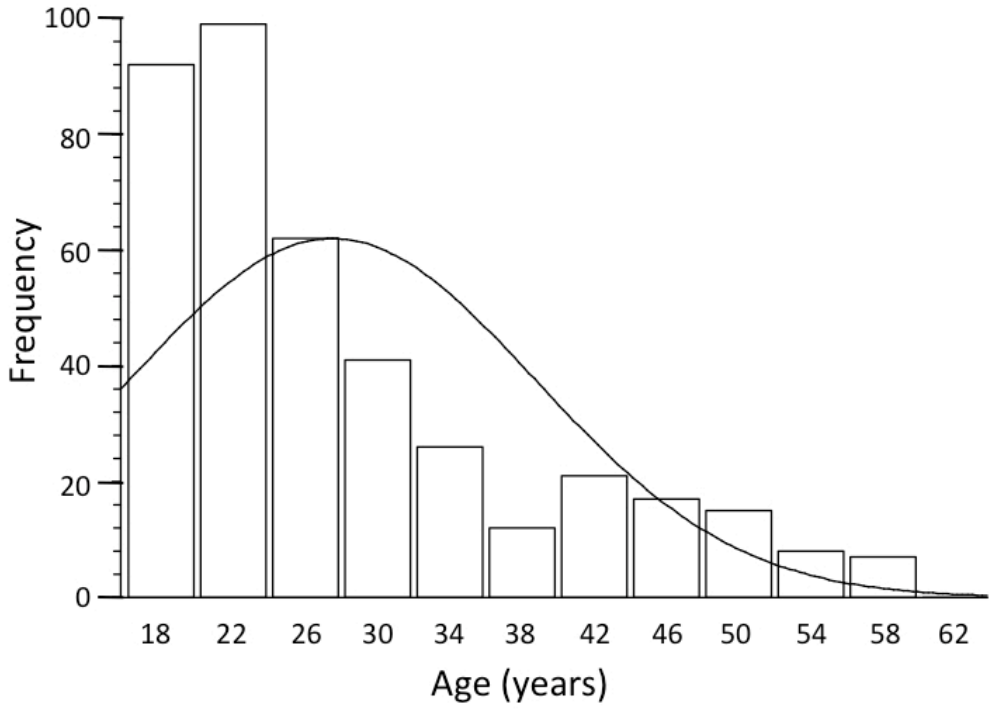


Figure 2.1 Age distribution of volunteers recruited to the HLA-typed cohort by CDSS nurses (n=400).

Volunteers from varied ethnic backgrounds were particularly sought, in order to increase the allelic diversity within the cohort. It is well known that some HLA associations have only been observed in certain populations (Alfirevic et

al., 2006). In order to maximise the scope of our cohort it was therefore important to include volunteers from backgrounds that have a higher frequency of these alleles. The ethnicities of volunteers recruited to the cohort are shown in Figure 2.2. The majority of volunteers were of a Caucasian background (84%).

The average number of lymphocytes isolated from each volunteer was 96 million ± 40 million, though not all volunteers donated the full 100ml.

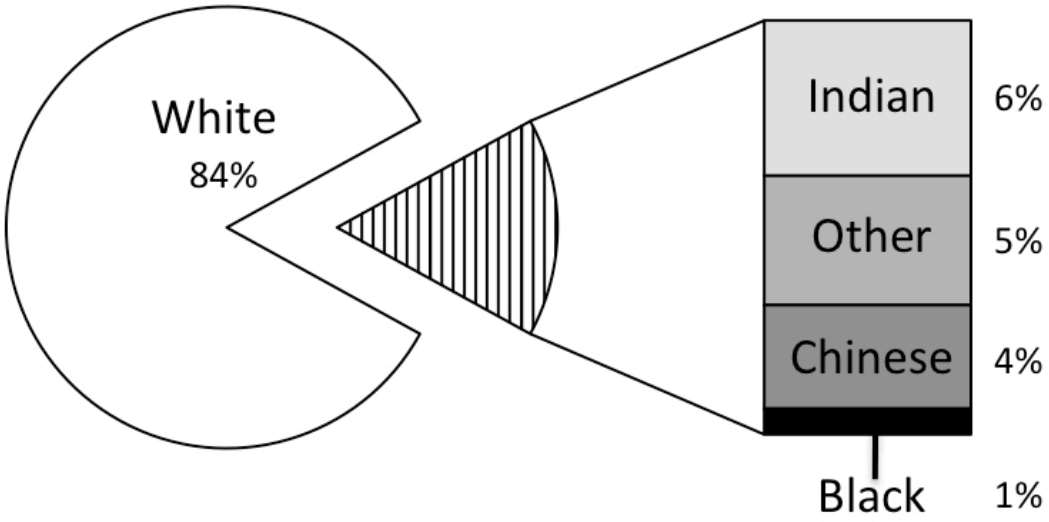


Figure 2.2 Ethnicities of volunteers recruited to the HLA-typed cohort n=400. Ethnicities were self-reported.

2.4.2 Allele frequencies

In our cohort 43 different HLA-A alleles were present, the most frequent of these being HLA-A*02:01 which was expressed by 176 individuals (23 homozygotes, 153 heterozygotes). As expected the HLA-B locus was the most

polymorphic allele in our population, in which 75 different alleles were present. The most common HLA-B allele was HLA-B*07:02 for which there were 9 homozygotes and 85 heterozygotes. For HLA-C, a total of 29 alleles were present the most prevalent of which was HLA-C*07:01 (9 homozygotes, 110 heterozygotes). At the DRB1 locus 42 alleles were present. The most common allele was DRB1*15:01:01 which was present in 110 individuals (10 homozygous, 100 heterozygous). Nineteen different HLA-DQB1 alleles were present, the most frequent of which was HLA-DQB1*02:01 which was carried by 163 individuals (21 homozygotes, 142 heterozygotes). For tables of all alleles present within the cohort see Appendix (Tables A1-A5).

Table 2.1 and 2.2 summarise the number of individuals expressing some of the HLA alleles that have been associated with a range of adverse drug reactions. Lymphocytes from several of these individuals were used in functional T-cell assays to determine the ability of the associated drugs to elicit a drug-specific effect (see Chapter 3).

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Table 2.1 HLA alleles associated with drug-induced hypersensitivity reactions and cell availability in the HLA-typed archive (n=385)

Drug	HLA allele	Reaction (Ethnicity)	Odds ratio (95% CI)	Reference	Number of carriers in the cohort	
					Hm	Ht
Drug-induced hypersensitivity reactions						
Abacavir	B*57:01	Hypersensitivity (all)	117 (29-481)	(Mallal et al., 2002)	1	25
Carbamazepine	B*15:02	SJS/TEN (Han Chinese)	2504 (126-49,522)	(Chung et al., 2004)	1	6
	A*31:01	All phenotypes (Caucasians)	12.12 (4.03-20.65)	(McCormack et al., 2011)	0	2
Allopurinol	B*58:01	SJS (Han Chinese) (Caucasian)	580.3 (34.4-9780.9) 80 (34-187)	(Hung et al., 2005, Lonjou et al., 2008)	0	6
Nevirapine	DRB1*01:01	Hypersensitivity (Caucasian)	4.8 (1.3-16.8)	(Martin et al., 2005)	3	52
Lamotrigine	B*38	SJS/TEN (Caucasian)	6.8 (2.6-18)	(Lonjou et al., 2008)	0	7

CI=Confidence Interval; ND= not determined, DQA1 locus not genotyped in ENW cohort; Hm=Homozygotes; Ht=Heterozygotes; SJS= Stevens Johnson syndrome; TEN= toxic epidermal necrolysis.

Table 2.2 HLA alleles associated with drug-induced liver injury and cell availability in the HLA-typed archive (n=385)

Drug	HLA allele	Reaction (Ethnicity)	Odds ratio (95% CI)	Reference	Number of carriers in the cohort	
					Hm	Ht
Drug-induced liver injury						
Flucloxacillin	B*57:01	DILI (Caucasian)	80.6 (22.8-284.9)	(Daly et al., 2009)	1	25
Ximelagatran	DRB1*07:01	DILI (Caucasian)	4.4 (2.2-8.9)	(Kindmark et al., 2007)	10	91
	DQA1*02:01		4.4 (2.2-8.1)		21*	142*
Lumiracoxib	DRB1*15:01	DILI (Caucasian)	5.3 (3.0 to 9.3)	(Singer et al., 2010)	10	100
Co-amoxiclav	DRB1*15:01	DILI (Caucasian)	2.3 (1.0 to 5.3)	(Donaldson et al., 2010, Lucena et al., 2011)	10	100
	A*02:01		2.2 (1.6-3.2)		23	153
Antituberculosis (isoniazid, rifampicin, pyrazinamide)	DQB1*02:01	DILI (Indian)	1.9 (1.0 to 3.9)	(Sharma et al., 2002)	20	143
Lapatinib	DQA1*02:01	DILI (Caucasian)	2.2 (1.1 to 5.7) 9.0(3.2 to 27.4)	(Spraggs et al., 2011)	21*	142*
Diclofenac	DRB1*13	DILI (Caucasian)	-	(Daly and Day, 2009)	-	-
Ticlopidine	A*33:03	DILI (Japanese)	13.0 (4.4 to 38.6)	(Hirata et al., 2008)	1	8

CI=Confidence Interval; ND= not determined, *DQA1 locus was not genotyped in ENW cohort so DQB1 is reported; Hm=Homozygotes; Ht=Heterozygotes; DILI= Drug-induced liver injury

2.4.3 Availability of volunteers for secondary donations

In August 2010 a small cohort of volunteers was approached on a second occasion in order to obtain a further blood donation to replenish lymphocyte stocks. Of 19 individuals that were recalled 15 (79%) were able to donate again. From the 4 that were unable to donate, 2 were pregnant and therefore ineligible to donate at that time, and 2 could not be contacted. This suggested that should further samples be required from particular individuals there was a good chance of volunteers being willing to donate again. It is however anticipated that given the size of the cohort there are sufficient numbers of individuals with interesting HLA alleles in order that alternative donors with the required genotype could be selected if necessary. In a more recent study conducted in Summer 2012, only 18 out of 64 volunteers were contactable (28%) indicating that the recall rate has fallen considerably over time.

2.4.4 HCP5 as a tagging SNP for HLA-B*57:01

Three individuals were found to possess a copy of the G allele out of 23 samples tested (Figure 2.3). Sequence-based typing confirmed that these individuals had the following HLA-B alleles: B*14:02/B*57:01, B*49:01/B*57:01, B*08:01/B*57:01.

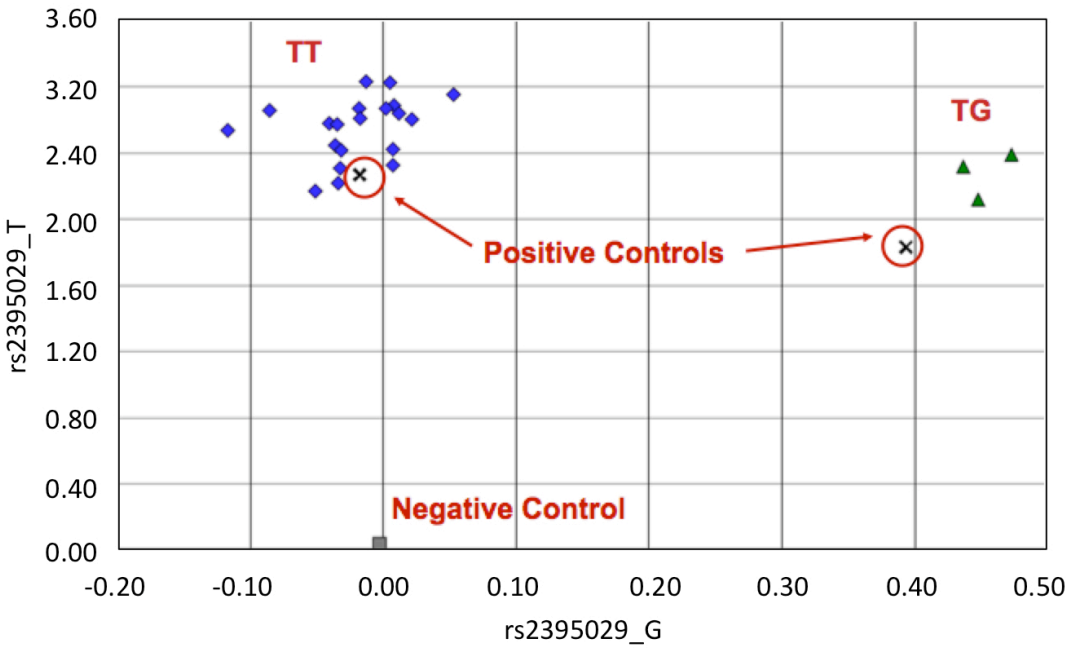

Figure 2.3 Allelic discrimination of rs2395029 in 23 volunteers. Positive controls represent individuals previously typed by sequence-based techniques. Negative control contains no DNA.

2.5 Discussion

Immune-mediated adverse reactions to drugs are incredibly difficult to predict, and much still remains to be established regarding their underlying mechanisms. Recently observed genetic associations have focussed attention on the MHC region and have implicated specific HLA alleles in the development of adverse reactions to certain drugs (Tables 2.1 and 2.2).

A number of hypotheses have been proposed to explain the ability of small molecules such as drugs to initiate an immune response against host cells. Nevertheless, each of these mechanisms highlights that a unique interaction between drug, T-cell receptor and MHC molecule is a key factor in the development of immune-mediated adverse reactions and as such the study of

HLA alleles represents a logical route to study the genetic basis of such reactions.

In order to further study these HLA-linked reactions *in vitro* it will therefore be important to work with genetically characterised cells. Immune cells bearing these HLA types can be utilised in functional assays to determine the propensity of small molecule drugs to initiate T-cell responses. This approach has already shown some success with regard to reactions to abacavir. Drug-specific secretion of interferon-γ can be detected in lymphocytes isolated from HLA-B*57:01 positive abacavir-naïve healthy donors, following a short priming period (Chessman et al., 2008). The creation of a DNA and cell archive will therefore be a valuable tool in further exploring the role of HLA molecules in the development of adverse drug reactions in certain susceptible individuals.

The current study has been successful in creating a biobank of both DNA and lymphocytes from 400 healthy volunteers in order to study HLA-associated ADRs. These volunteers are drawn from the North-West region and encompass a range of ethnicities. This was important to the study given that some HLA-associations are confined to specific ethnic groups (Alfirevic et al., 2006). In order to be able to study the involvement of rare alleles it was therefore important to recruit individuals from some of these backgrounds.

Advertisements were placed in local newspapers and on both university and hospital electronic announcement systems. In recruiting volunteers it was also important to ensure that volunteers would be contactable in the future. For this

reason the cohort is composed of individuals from the hospital staff and local residents in addition to university students who may not remain in the North-West once they have finished their studies.

The cells stored in our cell bank will be utilised in functional assays to determine the propensity of different drugs to elicit a T-cell response in lymphocytes from volunteers with a known genetic background. For most HLA-associated adverse drug reactions it has not yet been established that the HLA allele is indeed the causative allele. Where a prevalent HLA allele is reported to be associated with a reaction it is unlikely that this alone is responsible for the toxicity observed. It is likely that an extended haplotype exists in which other genes may be implicated. The complex interplay between these genes can only be replicated in systems where these haplotypes are present. Humanised animal models, in which specific human HLA alleles can be inserted into the mouse genome have previously been used to model autoimmunity (Shultz et al., 2007). However, given the great complexity in the MHC region in which linkage disequilibrium can confound the discovery of genetic associations and the likely role for specific HLA haplotypes, it is unclear how successful this approach would be in studying adverse drug reactions. Through the use of human lymphocytes in which these haplotypes remain intact it is hoped that a more complete picture of the mechanisms underlying drug immunogenicity will be created.

For many of the genetic associations observed it is likely that other factors will be involved in determining whether an individual experiences a reaction to a

particular drug. Even for the strongest associations there is not 100% correlation between genotype and phenotype. In the case of abacavir hypersensitivity only 55% of patients expressing the HLA-B*57:01 allele will develop a reaction if exposed to the drug (Mallal et al., 2008). Other factors, both genetic and environmental e.g viral infection, immune status of the individual are likely to be involved. Some of these environmental factors may not be reproducible *in vitro*, however it is hoped that the experimental strategy that has been adopted will be successful in detecting drug-reactive T-cells.

The availability of high-quality DNA from volunteers that have been HLA-typed by sequence-based methods also provided an opportunity to investigate a recently discovered tagging-SNP for HLA-B*57:01. This technique exploits the linkage disequilibrium displayed between HLA-B*57:01 and a T to G substitution located 100kB centromeric of the HLA-B locus allowing rapid PCR-based genotyping. A number of studies have been performed in order to determine whether this assay would be useful in assigning patient genotypes prior to abacavir prescription, with varying results. Colombo et al. (2008) performed HCP5 SNP genotyping in 1103 individuals that were also HLA-typed by sequence-based techniques. All HLA-B*57:01 positive individuals (n=98) were found to possess a copy of the HCP5 SNP (Colombo et al., 2008). Out of 1005 HLA-B*57:01 negative individuals, 999 were also negative for HCP5. Six individuals however tested positive for the HCP5 SNP but were not carriers of HLA-B*57:01. The authors suggest that though there is some discrepancy in the findings the relative cost and speed of genotyping by this method warrants further investigation, particularly for use in situations where sequence-based

typing techniques are not available. A study of 268 DNA samples from patients of Mexican Mestizo origin found complete agreement between HLA-B*57:01 status and carriage of the HCP5 SNP (Sanchez-Giron et al., 2011). The authors felt that the tagging SNP method would therefore be useful in a clinical setting as HLA-typing is not currently performed in Mexico due to the associated costs (Sanchez-Giron and Carnevale, 2012). In an Italian study of 185 patients, 5 discrepancies were observed. Three HLA-B*57:01 negative individuals were positive for the HCP5 SNP and more worryingly two HLA-B*57:01 positive individuals were negative for the HCP5 SNP (Badulli et al., 2012). This result is of particular concern as were typing by tagging SNP alone performed, these individuals could be exposed to the drug and therefore be at risk of experiencing a DHR. Taken together these studies suggest that HCP5 SNP genotyping is unlikely to be a replacement to the current gold-standard sequence-based techniques but that it may prevent DHRs in countries where no HLA-typing is currently performed.

The establishment of this cohort of HLA typed volunteers represents a hugely valuable resource in the study of adverse drug reactions. It provides a bank of lymphocytes from a known genetic background that can be used to further elucidate the mechanisms underlying the pathogenesis of HLA-associated ADRs. Utilising the outlined experimental strategy it is hoped that the involvement of HLA molecules in these reactions can be investigated fully.

Chapter 3

Development of *in vitro* assays to detect HLA-restricted T-cell responses

3.1 Introduction

The involvement of drug-specific T-cells in hypersensitivity reactions is well established. There are a number of methods currently available to detect or indeed prime drug-specific T-cell responses *in vitro*.

The lymphocyte transformation test (LTT) is commonly used to detect drug-specific T-cell responses *ex vivo* (Pichler and Tilch, 2004). Proliferation of peripheral blood mononuclear cells (PBMCs) isolated from hypersensitive patients in response to drug treatment signifies prior sensitisation and hence expansion of a memory-cell population. This proliferation does not occur in PBMCs from tolerant patients. The assay can be modified in order to reveal more information about the responding cells through the use of CFSE staining coupled with antibodies directed at cell surface markers e.g CD4 and CD8, CD45RO and CD45RA (Hanafusa et al., 2012). It can however be more difficult to detect circulating drug-specific T-cells in cases of drug-induced liver injury where the LTT is positive in just 26% of cases (Maria and Victorino, 1997). In addition to proliferation, cytokine secretion can also be detected and quantified. Amoxicillin induces the secretion of interferon-γ from PBMCs of hypersensitive patients but not tolerant controls (Rozieres et al., 2009). Cytokine readouts may be more appropriate where $CD8^+$ T-cells are implicated. Gene expression analysis can also reveal the presence of drug-specific T-cells. Previously, cells isolated from PPD- allergic volunteers were found to express an array of Th2 type genes compared to a regulatory gene profile in tolerant controls (Coulter et al., 2010). These changes were detectable following just 24h incubation. This type of study may therefore be more applicable to a high-throughput setting.

In naïve individuals a different approach must be taken. Primary stimulation of naïve cells requires multiple rounds of restimulation with the drug and autologous antigen-presenting cells in the presence of interleukin-2 (Engler et al., 2004). Activation can be further enhanced by the provision of co-stimulatory signals such as CD40 during the priming period (Sanderson et al., 2007). Priming assays utilising dendritic cells as antigen-presenting cells have also recently been developed (Faulkner et al., 2012). These assays have all utilised the drug metabolite sulfamethoxazole nitroso (SMX-NO) for which no individual susceptibility factors have yet been identified. The priming assay could however be extended to HLA-associated DHRs through the use of genotyped cells.

Chessman et al. (2008) detected abacavir-specific secretion of IFN-γ from PBMCs of drug-naïve HLA-B*57:01 positive donors. PBMCs were incubated with abacavir in the presence of IL-2 for 14 days and subsequently rechallenged with the drug. Cells isolated from donors expressing HLA-B*57:01 secreted IFN-γ and killed target cells in a dose-dependent manner. When antigen-presenting cells expressing other HLA-B alleles were substituted into the assay the response was blocked. The relatively short incubation time and seemingly high precursor frequency led the authors to hypothesise that these cells were pre-existing in individuals possessing the HLA risk allele. No evidence of any viral cross-reactivity was observed and TCRs were polyclonal. This may therefore represent a unique mechanism of activation in reactions where a HLA molecule is strongly associated with immunogenicity. This is supported by similar findings when PBMCs isolated from HLA-B*15:02 positive volunteers were treated with carbamazepine (Ko et al., 2011).

Gene expression analysis has previously been used to investigate the T-cell response of cells isolated from PPD- allergic volunteers (Coulter et al., 2010). The authors found that there were significant differences between the way that cells from allergic and tolerant individuals responded to incubation with PPD, in terms of cytokine mRNA profile. These changes were detectable following just 24h incubation. This type of study may therefore be more applicable to a high-throughput setting.

In this chapter ximelagatran, flucloxacillin and abacavir have been used as test compounds representing drugs associated with liver injury and drug hypersensitivity reactions. As described above abacavir has previously been shown to activate T-cells in a HLA-restricted manner and as such will act as a positive control in development of the assays. Flucloxacillin-induced liver injury is also associated with carriage of HLA-B*57:01 (Daly et al., 2009). The evidence for the involvement of T-cells in flucloxacillin-induced liver injury is however fairly minimal, with only a few drugs being associated with positive LTTs in patients (Maria and Victorino, 1997). Whether any genetic markers could be identified that are applicable to both forms of immune-mediated reaction is an interesting area to investigate. ALT elevations experienced during ximelagatran treatment are associated with expression of HLA-DRB*07:01 and positive LTTs implicate T-cells in the reaction (Kindmark et al., 2008). The role of the HLA molecule in the pathogenesis of the reaction has not yet been investigated.

3.2 Aims

- Confirm the findings of Chessman et al (2008) with regard to abacavir and apply the experimental strategy to investigate T-cell responses to ximelagatran
- Utilise genotyped cells to determine the propensity of ximelagatran, flucloxacillin and abacavir to elicit T-cell responses
- Investigate gene expression changes associated with abacavir and flucloxacillin treatment in cells from HLA-B*57:01 positive and negative individuals

3.3 Methods

3.3.1 Chemicals and reagents

Human AB serum was obtained from Innovative Research (Michigan, USA). Foetal bovine serum (FBS) was obtained from Invitrogen, Paisley, UK. Tetanus toxoid (TT) was bought from the Statens Serum Institut, Copenhagen, Denmark. RNeasy mini kit and RNase-free DNase kits were purchased from Qiagen, Crawley, UK. Custom-designed codesets and other NanoString reagents were all sourced from NanoString technologies, Seattle, USA. The RNA 6000 Nano kit was purchased from Agilent technologies, Stockport, UK. TaqMan primers were bought from Applied Biosystems, Warrington, UK, as were other PCR reagents including mastermix and reverse transcription reagents.

Interferon-γ, interleukin-13, interleukin-5, granzyme B and perforin ELISpot kits including antibodies and substrate solution were purchased from Mabtech,

Stockholm, Sweden. The Fas ligand ELISpot kit was bought from Abcam (Cambridge, UK). CD4-PE and CD8-FITC antibodies were purchased from BD Biosciences, Oxford, UK. T-cell receptor antibodies were obtained from AbD Serotec, Oxford, UK. Bio-Plex Pro Human Cytokine Th1/Th2 Panel was purchased from Bio-Rad, Hertfordshire, UK. Recombinant human interleukin-2 (rhIL-2) was supplied by Peprotech, London, UK. [^{3}H]-Thymidine was purchased from Moravek (California, USA). Multisort bead separation kits were bought from Miltenyi Biotec, Surrey, UK.

Abacavir sulfate and ximelagatran were kind gifts from GlaxoSmithKline and AstraZeneca respectively. Flucloxacillin was obtained from the Royal University Hospital, Liverpool. All other reagents were obtained from Sigma-Aldrich, Dorset, UK.

3.3.2 Cell culture medium

T-cell medium was comprised of RPMI 1640 supplemented with 10% human AB serum, 25mM HEPES, 1000U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-glutamine and 25µg/ml transferrin.

Antigen-presenting cell medium was comprised of RPMI 1640 supplemented with 10% foetal bovine serum, 25mM HEPES, 1000U/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine.

3.3.3 *In vitro* enrichment of drug-specific T-cells

3.3.3.1 Volunteer Characteristics

Table 3.1 indicates the HLA type of the 18 volunteers whose lymphocytes were used in the first investigations with abacavir and ximelagatran. All individuals were recruited to the HLA-typed cohort described previously (Chapter 2). Due to the linkage disequilibrium that exists between HLA-B*57:01 and HLA-DRB*07:01 three groups were investigated:

- Individuals expressing both HLA-B*57:01 and HLA-DRB*07:01
- Individuals just expressing HLA-DRB*07:01
- Individuals expressing neither HLA-B*57:01 nor HLA-DRB*07:01

Only one individual (155) expressed HLA-B*57:01 but not HLA-DRB*07:01. This sample was included within the first group.

Table 3.1 Genotypes of 18 volunteers from whom lymphocytes were used in this study

Volunteer ID.	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1
001	01:01/02:01	51:01/**57:01**	06:02/07:02	15:01/**07:01**	03:03/06:02
002	11:01/24:02	27:02/**57:01**	05:01/06:02	14:01/**07:01**	03:03/05:03
003	01:01/03:01	14:02/**57:01**	06:02/08:02	13:02/**07:01**	03:03/06:09
004	01:01/01:01	**57:01**/**57:01**	06:02/06:02	04:03/**07:01**	03:02/03:03
005	24:02/26:01	40:06/44:03	07:01/15:02	**07:01**/**07:01**	02:01/03:03
006	03:01/11:01	27:05/**57:01**	01:02/07:02	01:01/**07:01**	03:03/05:01
007	01:01/02:01	07:02/58:01	03:02/07:02	13:02/04:01	03:01/06:09
008	01:01/26:01	38:01/40:01	03:04/12:03	**07:01**/08:01	02:01/04:02
009	02:01/03:01	07:02/15:18	04:01/07:02	04:01/**07:01**	02:01/03:01
010	11:01/74:03	07:02/44:03	01:02/04:01	15:01/**07:01**	02:01/06:02
011	03:01/32:01	07:02/14:01	07:02/08:02	01:01/**07:01**	02:01/05:01
012	01:01/11:01	40:06/**57:01**	06:02/15:02	15:01/14:04	05:03/06:01
013	01:01/02:01	07:02/40:01	03:04/07:02	**07:01**/08:01	02:01/04:02
014	03:01/11:01	07:02/35:01	03:04/04:01	01:01/04:04	03:02/05:01
015	02:01/03:01	07:02/27:02	02:02/07:02	01:01/15:01	05:01/06:02
016	01:01/31:01	07:02/37:01	06:02/07:02	15:01/15:01	06:02/06:02
017	02:01/68:01	40:01/44:02	03:04/05:01	15:01/13:01	06:02/06:03
018	03:01/03:01	07:02/07:02	07:02/07:02	15:01/03:01	02:01/06:02

3.3.3.2 Lymphocyte transformation test (LTT)

The lymphocyte transformation test is designed to detect the presence of drug-specific memory T-cells in the peripheral blood of hypersensitive patients. PBMCs (1.5 x 10^5, 100µl) were incubated with abacavir (0-250µM, 100µl) or ximelagatran in triplicate wells for six days. Tetanus toxoid (0.5µg/ml) was included as a positive control. [^{3}H]- Thymidine (0.5µCi/well) was added for the final 16 hours of culture. Plates were harvested and proliferation was determined by scintillation counting. Stimulation indexes (SIs) were calculated by comparing proliferation in drug treated vs. control wells (SI= cpm in drug treated wells ÷ cpm in control wells). An SI> 2 was considered a positive result (Pichler and Tilch, 2004).

3.3.3.3 Lymphocyte stimulation

The method of Chessman *et al*. (2008) was used to expand and detect drug-reactive T-cells. Volunteer lymphocytes were quickly thawed, washed in HBSS and resuspended in complete T-cell medium. Cells were seeded in a 24 well plate (2 x 10^6; 1ml) with abacavir (35µM), ximelagatran (100µM) or tetanus toxoid (0.5µg/ml). Tetanus toxoid was used as a positive control as it is a peptide antigen that most of the UK population will have been immunised against. Cultures were supplemented with IL-2 (10U/ml) on days 3, 6, 9 and 11. On day 14 cells were harvested, counted and resuspended in fresh medium. Antigen specificity was determined via proliferation assay and cytokine secretion as described below.

3.3.3.4 Proliferation assay

Following lymphocyte stimulation 1 x 10^5 responder cells (50µl) were added to autologous irradiated PBMCs from frozen stocks (5 x 10^4, 50µl), which were used as antigen-presenting cells. Responder cells were restimulated with the same antigen as originally used during the 14-day stimulation (abacavir 10 and 35µM, ximelagatran 50 and 100µM, TT 0.1 and 0.5µg/ml). [^{3}H]-Thymidine (0.5µCi/well) was added after 48h and proliferation measured by scintillation counting. Dividing cells incorporate the radiolabelled thymidine into newly synthesised DNA. Stimulation indexes were calculated as described previously (see 3.3.3.2).

3.3.3.5 Enzyme-linked immunospot (ELISpot) assay

A schematic representation of the ELISpot assay is shown in Figure 3.1. On day 13 Multiscreen$_{HTS}$ filter plates (Millipore, Watford, UK) were coated overnight at 4˚C with interferon-γ capture antibody (15µg/ml). The following day, wells were washed five times with PBS and blocked with T-cell medium (200µl, 30min, room temperature). Responder cells (2 x 10^5, 50µl) were added to wells with autologous irradiated PBMCs as antigen-presenting cells (1 x 10^5, 50µl) and the antigen (100µl). Plates were incubated at 37˚C in 5% CO_2 and developed after 48h, according to the manufacturers instructions. At the end of the incubation cells were discarded and wells were washed five times with 200µl PBS. Biotin-labelled detection antibody was diluted to 1µg/ml in PBS containing 0.5% FBS and added to the wells (100µl). The plate was incubated at room temperature for 2 hours before wells were washed five times with PBS. Streptavidin-ALP was diluted 1:1000 in PBS containing 0.5% FBS and added to

wells (100µl, 1h, room temperature). Wells were washed five times with PBS (200µl) and spots were visualised by the addition of BCIP/NBT substrate (100µl, 15 min). The plate was counted on an AID ELISpot reader (Cadama Medical, Stourbridge, UK) when thoroughly dried.

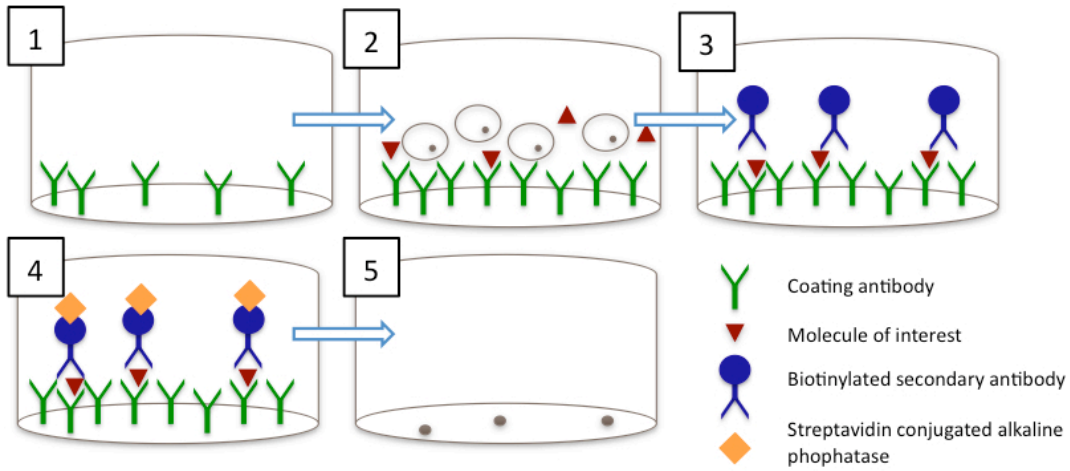

Figure 3.1 Schematic representation of the enzyme-linked immunospot (ELISpot) assay. Wells are coated with antibody specific for the molecule of interest (1). Cells are incubated in the plate for 48h and the molecule of interested is captured (2). The cells are removed and a biotinylated secondary antibody also specific for the molecule of interest is added (3). Streptavidin-conjugated alkaline phosphatase binds to the secondary antibody (4) and on addition of the substrate solution alkaline phosphatase catalyses a colour change reaction resulting in the formation of distinct dark spots (5).

3.3.4 Gene expression analysis

3.3.4.1 Volunteer characteristics

PBMCs from six volunteers were utilised in this part of the study. The HLA type of each individual is shown in Table 3.2

Table 3.2 HLA type of volunteers from whom PBMCs were isolated.

Volunteer ID.	HLA-A	HLA-B	HLA-C	HLA-DRB	HLA-DQB
004	01:01/01:01	57:01/**57:01**	06:02/06:02	04:03/07:01	03:02/03:03
019	01:01/02:01	08:01/**57:01**	06:02/07:01	03:01/03:01	02:01/02:01
020	02:01/11:01	44:02/**57:01**	05:01/07:01	04:02/07:01	03:02/03:03
021	01:01/02:01	07:02/08:01	07:01/12:03	15:01/03:01	02:01/06:02
022	02:01/66:01	15:03/44:02	05:01/12:03	03:01/03:01	02:01/02:01
023	01:01/02:01	08:01/44:02	05:01/07:01	03:01/04:01	02:01/03:01

3.3.4.2 T-cell stimulation

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from the blood of three HLA-B*57:01 positive and three HLA B*57:01 negative healthy volunteers via density centrifugation (as described in 2.3.3). Abacavir (50μM), flucloxacillin (1mM) or tetanus toxoid (5μg/ml) was added to PBMCs cultured in T-cell medium (final volume= 2ml; 5 x10^6 cells/condition). Cells were harvested at 2, 24 and 72h. After washing in HBSS, cell pellets were frozen and stored at -80˚C until RNA was isolated.

3.3.4.3 RNA isolation

Total RNA was extracted using the RNeasy mini kit. Lysis buffer (350μl) was added to the cell pellet. The lysate was passed through a 21g needle and an equal volume of 70% ethanol was added. After gentle pipetting the lysate was

applied to an RNeasy spin column. The spin column contains a silica membrane, which captures RNA. The column was centrifuged at >10,000g for 15 seconds and the flow-through discarded. On-column DNA digestion was performed utilising the RNase free DNase kit. DNase (80µl) was added directly to the column and incubated at room temperature for 15 minutes. The membrane was then washed with wash buffer RW1 (700µl) included in the kit. The membrane was then washed twice with RPE buffer, which contains a high concentration of ethanol. RNA was finally eluted with DNase/RNase free water (30µl). RNA was quantitated from a 1.5µl aliquot using the NanoDrop N-1000 spectophotometer (Thermo scientific, Surrey, UK). Nucleic acids absorb light with a characteristic wavelength of 260nm. Absorbance at 280nm is often indicative of protein contamination whereas absorption at 230nm is often as a result of contaminants from the extraction procedure. The A_{260}/A_{230} and A_{260}/A_{280} ratios therefore give an indication of the quality of RNA.

Integrity of the RNA was assessed using the Agilent Bioanlayser (Agilent, Stockport, UK), which employs microfluidics and electrophoresis in order to separate the RNA fragments in the sample based on charge and size. The sample (1µl) is compared against a reference ladder containing RNA fragments with known sizes (0.2kB, 0.5kB, 1.0kB, 2.0kB, 4.0kB, 6.0kB), which is run on each chip. The ratio of bands for 18S and 28S ribosomal RNA are compared to give an indication of the degree of degradation that is present in the sample. The software calculates an RNA integrity number (RIN), which can be used to compare samples.

3.3.4.4 NanoString

The expression of a panel of 46 genes was assessed using the Nanostring nCounter gene expression assay (see Table 3.3). The NanoString technology is a recently developed platform with a medium throughput capacity. It relies on hybridisation of fluorescently labelled probes to the target RNA transcript without the need for any thermal cycling. Up to 3000 genes of interest can be investigated in a single tube.

Gene name	Protein	Description
CCR1	Chemokine (C-C motif) receptor 1	Differentially expressed on immune cells. Most cells express several different chemokine receptors so that they can respond to different stimuli. CCR1-5 expressed on T cells, CCR2 and 5 expressed on B-cells. Dendritic cells express CCR1, 4 and 5.
CCR2	Chemokine (C-C motif) receptor 2	
CCR3	Chemokine (C-C motif) receptor 3	
CCR4	Chemokine (C-C motif) receptor 4	
CCR5	Chemokine (C-C motif) receptor 5	
CCL3	Chemokine (C-C motif) ligand 3	Cytokines mediating migration towards inflammation. Synthesised or released at the site of inflammation controlling the migration of specific cell types to the affected area. Induced by cytokines such as IFN-γ and TNF-α.
CCL4	Chemokine (C-C motif) ligand 4	
CCL5	Chemokine (C-C motif) ligand 5	
CCL7	Chemokine (C-C motif) ligand 7	
CCL11	Chemokine (C-C motif) ligand 11	
CXCR3	Chemokine (C-X-C motif) receptor 3	Chemokine receptor
CXCR4	Chemokine (C-X-C motif) receptor 4	Chemokine receptor
CX3CL1	Chemokine (C-X-C motif) ligand 1	Chemokine mediating cellular migration
CD4	CD4 molecule	Co-receptor in MHC-TCR interaction
CD80	CD80 molecule	Co-receptor in MHC-TCR interaction
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	Promotes the differentiation of haemopoietic stem cells to granulocytes and macrophages
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	High-affinity inhibitory receptor. Interacts with CD80 and CD86 on antigen presenting cells
CD40	CD40 molecule	Co-receptor in MHC-TCR interaction

Gene name	Protein	Description
CD40L	CD40 ligand	Co-receptor in MHC-TCR interaction. Binds to CD40
IL-1R1	Interleukin 1 receptor, type I	Cytokine receptor, binds IL-1α, IL-1β and IL-1RA
IL-2	Interleukin 2	Proliferation, promotes IFN-g secretion and antibody production
IL-4	Interleukin 4	Proliferation, promotes Th2 differentiation and IgG/IgE switching
IL-4R	Interleukin 4 receptor	Cytokine receptor, binds IL-4 and IL-13
IL-5	Interleukin 5 (colony-stimulating factor, eosinophil)	Proliferation and activation. Characteristic of Th2
IL-6	Interleukin 6	Lymphocyte growth, B cell differentiation
IL-7	Interleukin 7	Cellular division
IL-8	Interleukin 8	Monocyte and neutrophil activation/chemotaxis
IL-9	Interleukin 9	Division and development of T-cells and mast cells
IL-10	Interleukin 10	Secreted from Th2 cells. Inhibits cytokine synthesis.
IL-12A	Interleukin 12 A	Forms a heterodimer with IL-12B. Released by macrophages, activates NK cells
IL-12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	Forms a heterodimer with IL-12A. Released by macrophages, activates NK cells
IL-12RB2	Interleukin 12 receptor, beta 2	Cytokine receptor. Found on activated T-cells.
IL-13	Interleukin 13	Division and differentiation of B cells. Reduces cytokine production
IL-15	Interleukin 15	Division of T and B cells. Released from monocytes.
IL-17A	Interleukin 17 A	Increases chemokine production. Recruitment of monocytes and neutrophils.

Genes analysed via NanoString

Gene name	Protein	Description
IL-18	Interleukin 18 (interferon-gamma-inducing factor)	Induces IFN-γ and NK cell activity
IL-18R1	Interleukin 18 receptor	Cytokine receptor
ICOS	Inducible T-cell co-stimulator (CD278)	Co-stimulatory molecule
IFNG	Interferon-gamma	Released by activated Th1 cells. Antiviral. Enhances MHC expression. Increases IL-2 receptors.
INHBA	Inhibin beta a	Lymphocyte differentiation
LAG3	Lymphocyte-activation gene 3	Expressed in activated T and NK cells
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9 (CD137)	Expressed on activated T-cells, especially $CD8^{+}$. Co-stimulatory molecule.
GATA3	GATA binding protein 3	T-cell specific transcription factor
RPL19	60S ribosomal protein 19	Housekeeping gene
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Housekeeping gene
GUSB	Glucuronidase beta	Housekeeping gene

Total RNA (150ng) was incubated with the reporter and capture probe sets overnight at 65˚C. Twelve samples can be analysed on each run. A mastermix comprising 130µl reporter probe and 130µl hybridisation buffer was prepared. A 12-tube strip of PCR tubes was labelled and 20µl of the mastermix was added to each tube. RNA (5µl) was then added and mixed by gently flicking the tubes. Finally the capture probe (5µl) was added to tubes and mixed. The tubes were then immediately transferred to a heat-block set at 65˚C.

Both probes are specific for the gene of interest. Each reporter probe is coupled to a unique colour-coded sequence at the 5' end and the capture probe has a biotin label at the 3' end. The following day a number of washes to remove excess unbound probes are performed in an automated prep station. Where hybridisation has occurred a tripartite complex is formed comprising reporter probe, target mRNA and capture probe. These tripartite complexes are immobilised on streptavidin-coated slides. An electric current is applied to the slides in order to orientate the fluorescent labels and facilitate counting. Slides are imaged and counted on the nCounter digital analyser.

The unique fluorescent label of each gene is quantified in each sample. Counts for each gene are then downloaded for subsequent analysis. Normalisation to spiked-in positive controls is first performed in order to account for any differences in hybridisation efficiency. Data was further normalised for amount of RNA based on the expression of the housekeeping genes GUSB, RPL19 and HPRT1. All analyses were performed in nCounter software. The expression of each gene was compared between control vs. treated samples in HLA-B*57:01

positive and HLA-B*57:01 negative groups. Fold change between averaged treated and control samples was also determined.

3.3.4.5 TaqMan real-time PCR

In order to further validate these data, a smaller subset of 5 genes was selected for confirmatory real-time PCR (CCL3, CXCR3, IL-1R1, IL-6, IL-8). Genes were selected based on the results of the NanoString assay. Genes were selected where a fold change of greater than 2 was observed between averaged control and treated values. The 24-hour time-point was selected for further investigation.

Unlike the NanoString, TaqMan real-time PCR must be performed with cDNA. RNA (900ng) was therefore reverse transcribed using the high capacity RNA to cDNA mastermix. The mastermix contains all of the components required for efficient reverse transcription ($MgCl_2$, dNTPs, RNase inhibitor, reverse transcriptase, random primers and oligo(dT) primers). Mastermix (6µl; total volume 30µl) was added to the RNA and heated to 25˚C for 5 minutes. This was followed by 30 minutes at 42˚C and 5 minutes at 85˚C.

Each PCR reaction contained the following: 1.7µl cDNA, 10µl gene expression mastermix, 1µl gene expression assay and 6.3µl DNase-free water. Reactions were performed in a 384 well plate and all samples were run in duplicate. Blank water samples were included for each gene analysed. Thermal cycling was performed on an ABI 7900HT machine (Applied Biosystems, Warrington, UK). Reactions were heated to 95˚C for 10 minutes. This was followed by 50 cycles of

95˚C for 15 seconds, 60˚C for 1 minute. The TaqMan technology employs sequence-specific probes and primers. Probes are labelled with a fluorescent reporter dye such as FAM or VIC at the 5' end and a quencher at the 3' end. When the probe is bound to single-stranded DNA the quencher is sufficiently close to the fluorophore that no fluorescence is emitted. During extension of the complementary RNA chain Taq polymerase cleaves the probe, freeing the fluorescence from the quencher. This results in an increase in fluorescent intensity, which is proportional to the amount of target that has been amplified. The primers identify the area of the transcript to be amplified. The amount of fluorescence during each cycle is quantified and displayed graphically. Relative quantification is achieved by determining the cycle at which the fluorescence reaches a threshold value (C_t). The difference between the C_t of the gene of interest and the C_t of a housekeeping gene is then calculated for each sample (ΔC_t). This value is then compared between control and treated samples ($\Delta\Delta C_t$). Fold change is calculated by the following equation: Fold change = $2^{(-\Delta\Delta C_t)}$

3.3.4.6 Statistical analysis

The t-test was used to compare HLA-B*57:01 positive to negative or treated to untreated values.

3.3.5 T-cell cloning

3.3.5.1 Generation of abacavir-specific T-cell clones

The aim of T-cell cloning is to generate antigen-specific cultures derived from a single cell. PBMCs ($1x10^6$; 500µl) were cultured with abacavir (50µM) in T-cell medium. Cultures were supplemented with IL-2 (Peprotech, UK) on days 6 and 9. On day 14 $CD4^+$ and $CD8^+$ cells were separated using a CD8 MultiSort kit. Cells were harvested and resuspended in PBS containing 0.5% BSA and 2mM EDTA. Magnetic CD8 microbeads were added to the cells and incubated for 15 minutes at 4˚C. The cells were then washed twice and added to an MS column held in a magnet. Negative, unlabelled cells pass through the column and were collected as the $CD4^+$ fraction. The column was then removed from the magnet and the $CD8^+$ cells were flushed out. Purity of the cell populations was determined by FACS analysis. FITC-labelled CD8 and PE-labelled CD4 antibodies (3µl each) were added to an aliquot of the separated cells and incubated in the dark for 20 minutes at 4˚C. Events were collected on a BD FACS Canto II flow cytometer (BD Biosciences, Oxford). Representative histograms are shown in Figure 3.2. Purity was generally >95%.

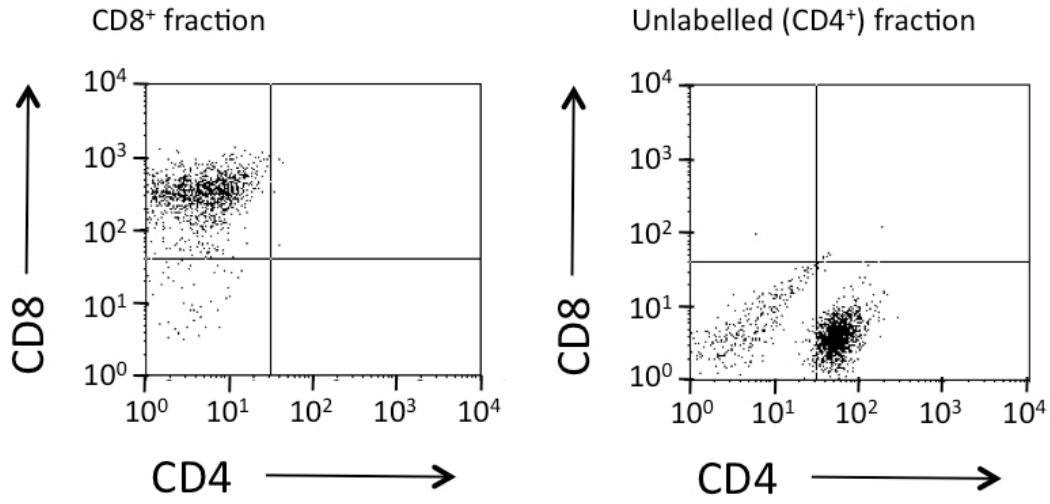

Figure 3.2 Purity of cell fractions following magnetic bead separation. A positive selection for $CD8^+$ cells was performed given the HLA class I association.

Separated cells were then cloned by serial dilution using established methods (Wu et al., 2006). Cells were seeded in 96-well plates at densities of 0.3, 1 and 3 cells/well and restimulated with allogenic irradiated PBMCs (5 x 10^4 cells/well), PHA (1µg/ml) and IL-2 (60U/ml). Well-growing clones were transferred to new plates and split as required. Clones were restimulated every 14 days as described above in order to maintain T-cell proliferation.

3.3.5.2 Generation of autologous antigen-presenting cell lines

Epstein-Barr virus (EBV) transformed B-cell lines were created from PBMCs by transformation with supernatant from the virus-producing cell line B9.58. Supernatant (5ml) was filtered through a 0.45µm syringe filter on to a pellet of 5 x 10^6 PBMC. Cyclosporin A (5µl) was added in order to inhibit the proliferation of T-cells. After overnight incubation at 37˚C, cells were washed and resuspended in antigen-presenting cell medium supplemented with CSA

(1µg/ml) and transferred to a 24-well plate. Fresh medium was added twice a week and CSA was omitted from the medium after two weeks in culture. Cells were transferred to a tissue culture flask when confluent.

3.3.5.3 Antigen-specificity of T-cell clones

At least 28 days after the serial dilution, abacavir-specificity was assessed by the addition of autologous irradiated EBV-transformed B-cells (1 x 10^4/well) and abacavir (50µM) to T-cell clones (5 x 10^4/well; 200µl). [^{3}H]-Thymidine (0.5µCi) was added after 48h and proliferation measured by scintillation counting. Clones with a stimulation index of greater than 2 were expanded by repetitive mitogen stimulation in IL-2 containing medium for further analysis. The effect of drug concentration on the T-cell response was measured by incubating clones (5 x10^4) and irradiated EBV-transformed B-cells (1 x 10^4) with abacavir (1, 5, 10, 50, 100µM). The response was quantified via both proliferation and IFN-γ ELISpot assays as described previously (see 3.3.3.4 and 3.3.3.5).

3.3.5.4 T-cell receptor Vβ phenotyping

T-cell clones were incubated with 30 different TCR Vβ antibodies in a 96-well V bottomed plate. All clones were also stained with APC-labelled CD8 antibody. T-cell clones were resuspended in FACS buffer (HBSS containing 0.5% BSA and 2mM EDTA) and incubated with the antibodies for 20 minutes in the dark at 4˚C. The different fluorescent markers meant that up to 3 antibodies could be added to each well (1 x FITC, 1x PE and 1x APC; all 3µl). The wells were then washed by centrifuging the plate for 5 minutes (600g, 4˚C). This was repeated a total of three times. Finally the cell pellets were resuspended in 300µl FACS

buffer and transferred to FACS tubes for analysis on the BD FACS Canto flow cytometer.

3.3.5.6 Secretion of cytokines and cytotoxic molecules

Secretion of IL-13, IL5, granzyme B, perforin and Fas ligand was determined by ELISpot assay. The general protocol was as described previously (3.3.3.5). Coating antibodies were used at a final concentration of 10μg/ml (IL-13) 15μg/ml (IFN-γ, IL-5, GrzB) or 30μg/ml (Perforin) depending on the analyte. The detection antibody was used at a concentration of 1μg/ml for all kits. The Fas ligand kit was obtained from a different supplier and so a number of changes were made to the protocol; coating antibody was diluted 1:100 in PBS, blocking was performed with 2% (w/v) skimmed milk for 2h, washes were performed with PBS containing 0.1% (v/v) tween-20, detection antibody was diluted 1:100 in PBS containing 1% BSA and incubated for 90 minutes at 37˚C and streptavidin-ALP was diluted 1:5000 and incubated for 1h at 37˚C.

Secretion of IL-2, IL-4, IL-5, IL-9, IL-10, IL-12, IL-13, GMCSF, IFN-γ and TNF-α was determined by Luminex multiplex bead assay. The Luminex assay allows the simultaneous quantification of multiple cytokines in the same sample (Figure 3.3). Colour-coded beads coupled to antibodies directed at each of the target molecules are incubated with the sample. Each antibody is coupled to a different bead with a unique colour. A fluorescently labelled reporter, also specific for the target is later added to quantify the amount of analyte bound to each bead. The Luminex reader detects both the colour of the bead and the amount of analyte bound.

Samples comprised cell culture supernatants. Eight T-cell clones (5 x 10^4) were incubated with and without 50μM abacavir in the presence of autologous irradiated EBV-transformed B-cells (1 x 10^4). After 48h 100μl supernatant was removed from the well and frozen at -80˚C until analysis. On the day of the assay samples were thawed on ice and diluted (1:2, final volume 120μl) with sample buffer. A single vial of standards was reconstituted in 500μl T-cell medium and diluted as shown in Table 3.4.

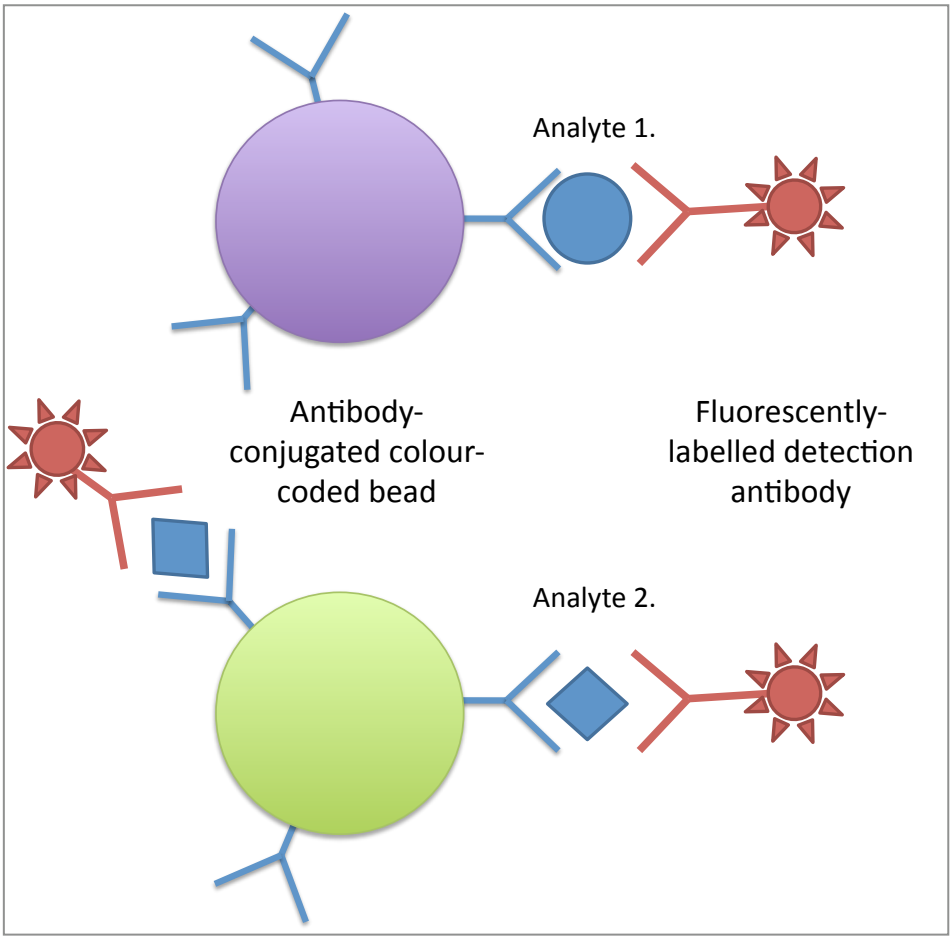

Figure 3.3 Principle of the Luminex assay. Colour-coded beads coupled to antibodies directed at each of the target molecules are incubated with the sample. Each antibody is coupled to a different bead with a unique colour allowing multiple analytes can be investigated in the same sample.

Table 3.4 Serial dilution of Luminex standards.

	Diluent	Standard
S1	72µl	128µl reconstituted standard
S2	150µl	50µl of S1
S3	150µl	50µl of S2
S4	150µl	50µl of S3
S5	150µl	50µl of S4
S6	150µl	50µl of S5
S7	150µl	50µl of S6
S8	150µl	50µl of S7

(S1: IL2 = 15,000pg/ml; IL-4 = 3500pg/ml; IL-5 = 32,000pg/ml; IL-9 = 10,000 pg/ml; IL-10 = 28,000pg/ml; IL-12 = 36,000pg/ml; IL-13 = 32,000pg/ml; GM-CSF = 13,000pg/ml; IFN-γ = 30,000pg/ml; TNF-α = 35,000pg/ml).

The filter plate was prewet with 100µl assay buffer. Magnetic beads were then added to the wells (50µl) and washed twice with wash buffer (100µl). Diluted samples and standards were then added to the plate (50µl) in duplicate and incubated in the dark for 30 minutes on a plate shaker. Detection antibody was diluted 1:10 in detection antibody diluent and added to wells (25µl). The plate was returned to the shaker for a further 30 minutes in the dark. After washing twice with wash buffer, Streptavidin-PE (diluted 1:100 in assay buffer) was added to wells (50µl). The plate was returned to the shaker for a further 10 minutes in the dark. Wells were washed three times with wash buffer, the beads were resuspended in 125µl assay buffer and the plate was read.

3.3.5.7 Statistical analysis

The Mann-Whitney test was used to compare proliferation in drug treated vs. control wells.

3.4 Results

3.4.1 *In vitro* enrichment of drug-specific T-cells

3.4.1.1 Lymphocyte transformation test

In order to determine prior sensitisation to the drugs investigated, the lymphocyte transformation test was performed. No positive proliferative responses were observed to either abacavir or ximelagatran (Figure 3.4). In contrast robust proliferative responses to tetanus toxoid were observed (SI=12-94).

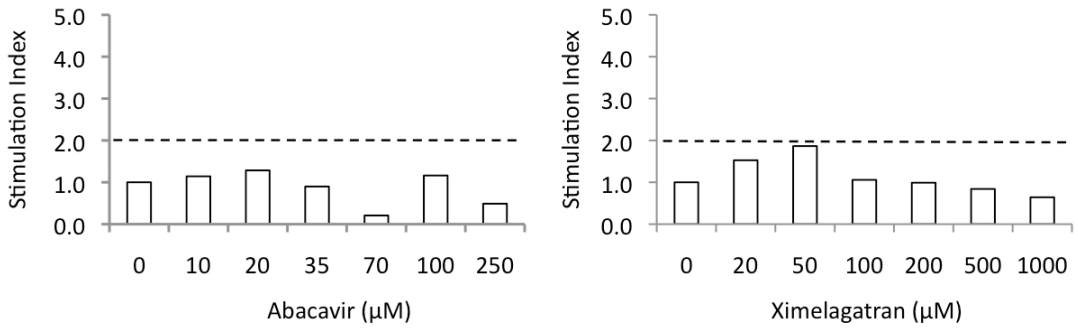

Figure 3.4 Representative lymphocyte transformation tests from a HLA-B*57:01/ HLA-DRB*07:01 positive volunteer. PBMC were incubated with the drug for 5 days and proliferation determined by [^{3}H]-thymidine incorporation. A stimulation index of >2 (indicated by the dashed line) were considered a positive result. The stimulation index for TT in this volunteer was 39.

3.4.1.2 Lymphocyte stimulation

Following 14-day incubation with either abacavir, ximelagatran or tetanus toxoid, T-cells were harvested and restimulated with the antigen of interest. Proliferation was determined by [^{3}H]-thymidine incorporation. Figures depict proliferative responses of lymphocytes expressing both HLA-B*57:01 and HLA-DRB*07:01 (Figure 3.5), lymphocytes with HLA-DRB*07:01 but not HLA-B*57:01 (Figure 3.6) and lymphocytes expressing neither allele (Figure 3.7). Robust proliferative responses were detected against the positive control tetanus toxoid in all volunteers (black bars). Abacavir stimulated a weak proliferation of T-cells isolated from volunteers 002 (SI= 2.3) and 004 (SI =2.0) (Figure 3.5; white bars). However this was only observed at a single concentration of abacavir (10μM). A very weak increase in proliferation was induced in T-cells from volunteer 005 who did not express HLA-B*57:01 (Figure 3.6; SI = 1.5 and 1.8 at 10 and 35μM abacavir respectively), however this was below the usual cut-off value of 2.0 (Pichler and Tilch, 2004). Ximelagatran induced very weak proliferation in lymphocytes isolated from volunteer 004 (SI=1.9) (Figure 3.5; grey bars). However, again this was below the cut-off value of 2.0 (Pichler and Tilch, 2004).

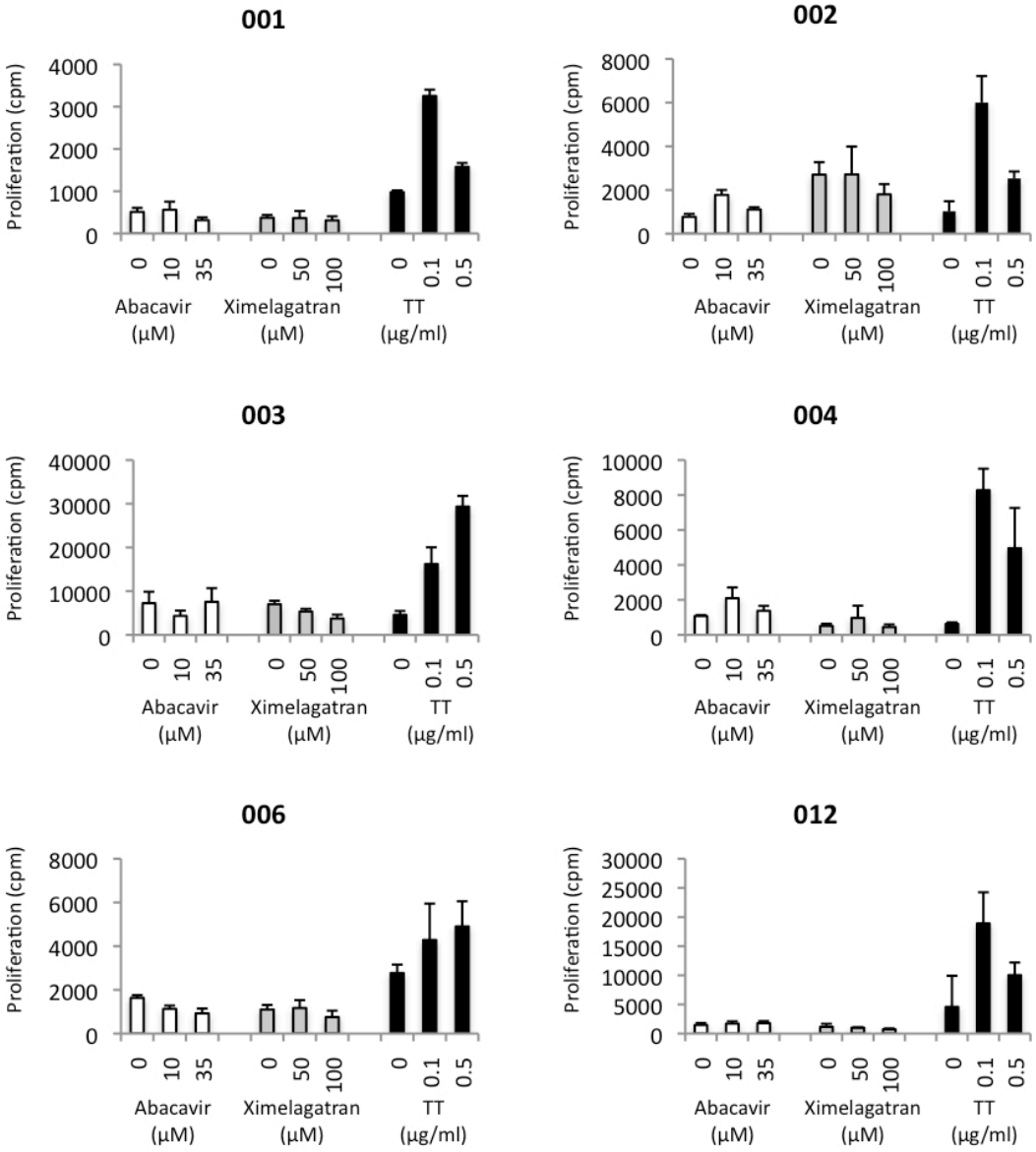

Figure 3.5 Antigen-specific proliferation of T-cells following 14-day enrichment period. HLA-B*57:01 positive donors. Following 14 day incubation with either abacavir, ximelagatran or tetanus toxoid T-cells were harvested and restimulated with the antigen of interest. Proliferation was determined by [^{3}H]-thymidine incorporation. Data represent mean and standard deviation of triplicate wells. TT= Tetanus toxoid.

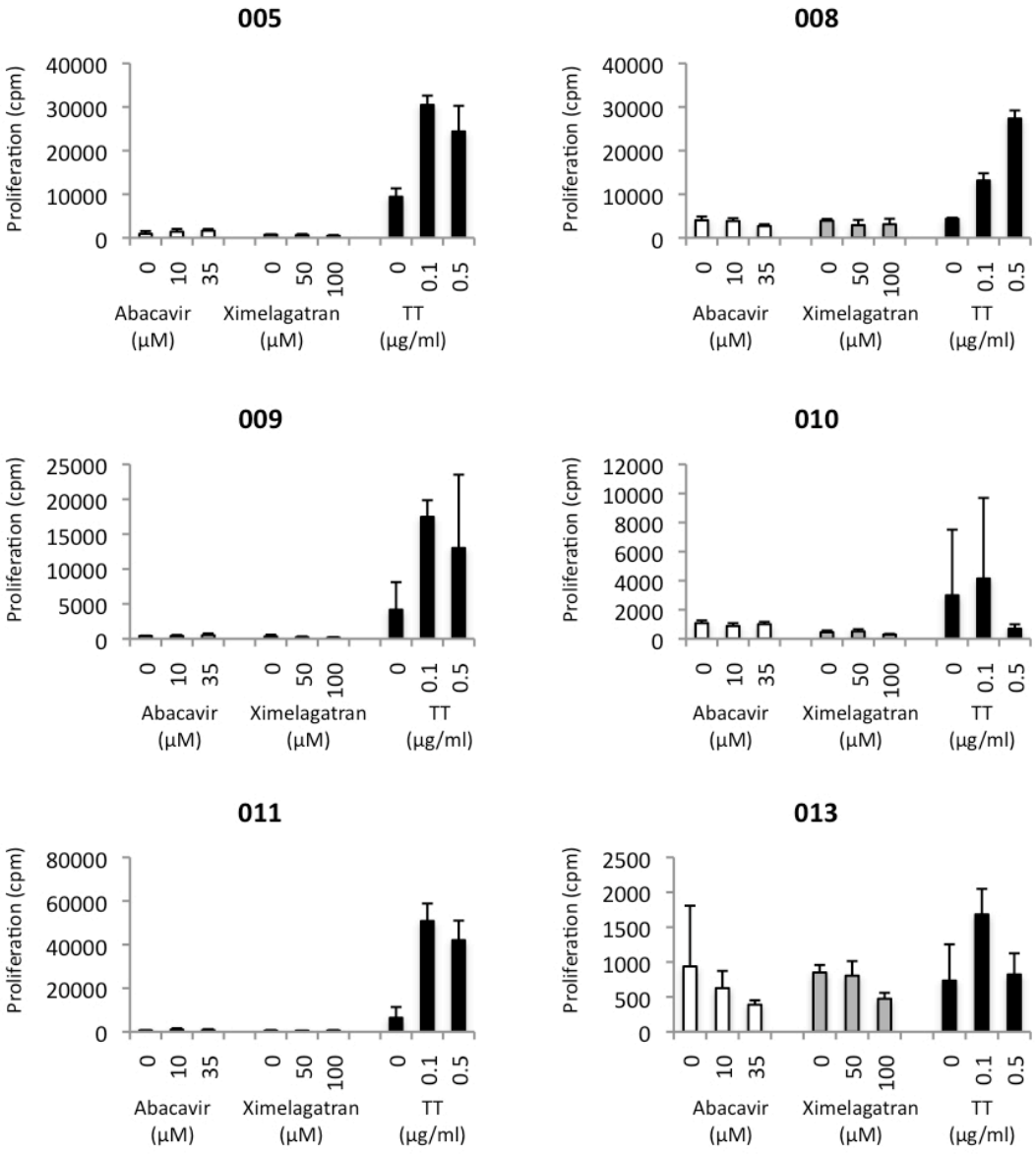

Figure 3.6 Antigen-specific proliferation of T-cells following 14-day enrichment period. HLA-DRB*07:01 positive, HLA-B*57:01 negative donors. Following 14 day incubation with either abacavir, ximelagatran or tetanus toxoid T-cells were harvested and restimulated with the antigen of interest. Proliferation was determined by [^{3}H]-thymidine incorporation. Data represent mean and standard deviation of triplicate wells. TT= Tetanus toxoid.

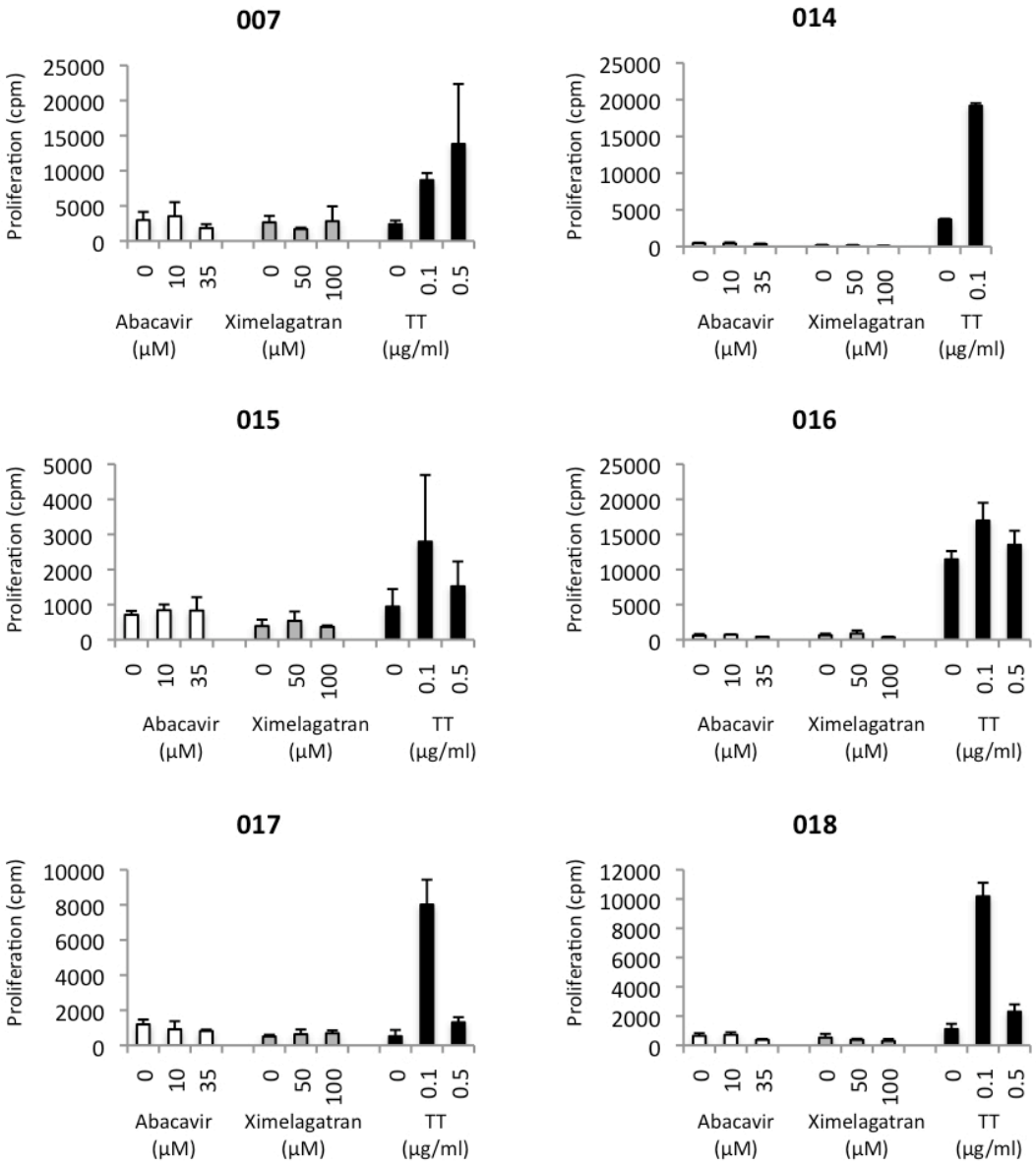

Figure 3.7 Antigen-specific proliferation of T-cells following 14-day enrichment period. HLA-DRB*07:01 negative, HLA-B*57:01 negative donors. Following 14 day incubation with either abacavir, ximelagatran or tetanus toxoid T-cells were harvested and restimulated with the antigen of interest. Proliferation was determined by [^{3}H]-thymidine incorporation. Data represent mean and standard deviation of triplicate wells. TT= Tetanus toxoid.

The second endpoint investigated was secretion of interferon-γ, determined by ELISpot assay. Figures depict cytokine secretion from lymphocytes expressing both HLA-B*57:01 and HLA-DRB*07:01 (Figure 3.8), lymphocytes with HLA-DRB*07:01 but not HLA-B*57:01 (Figure 3.9) and lymphocytes expressing

neither allele (Figure 3.10). Abacavir stimulated increased secretion of IFN-γ from lymphocytes isolated from volunteers 005 (SI=2.0; Figure 3.9) and 013 (SI= 1.7; Figure 3.9). Neither of these volunteers expressed HLA-B*57:01. Ximelagatran did not induce cytokine secretion of greater than 1.2 times greater than background level in lymphocytes from any volunteer.

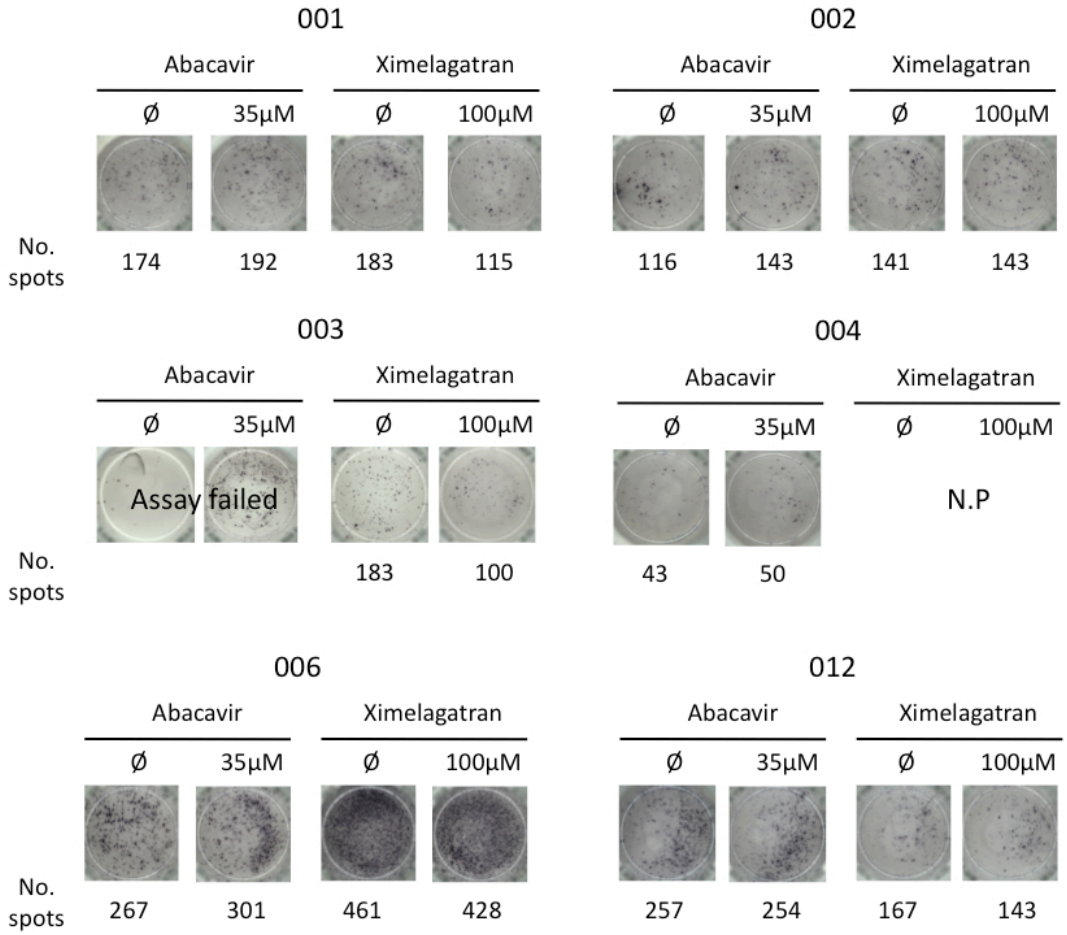

Figure 3.8 Drug-specific secretion of interferon-γ from T-cells following 14-day enrichment period. HLA-B*57:01 positive volunteers. Following 14-day incubation with either abacavir or ximelagatran T-cells were harvested and restimulated with the antigen of interest. Well images are shown with spot counts assigned by AID Software. N.P = not performed.

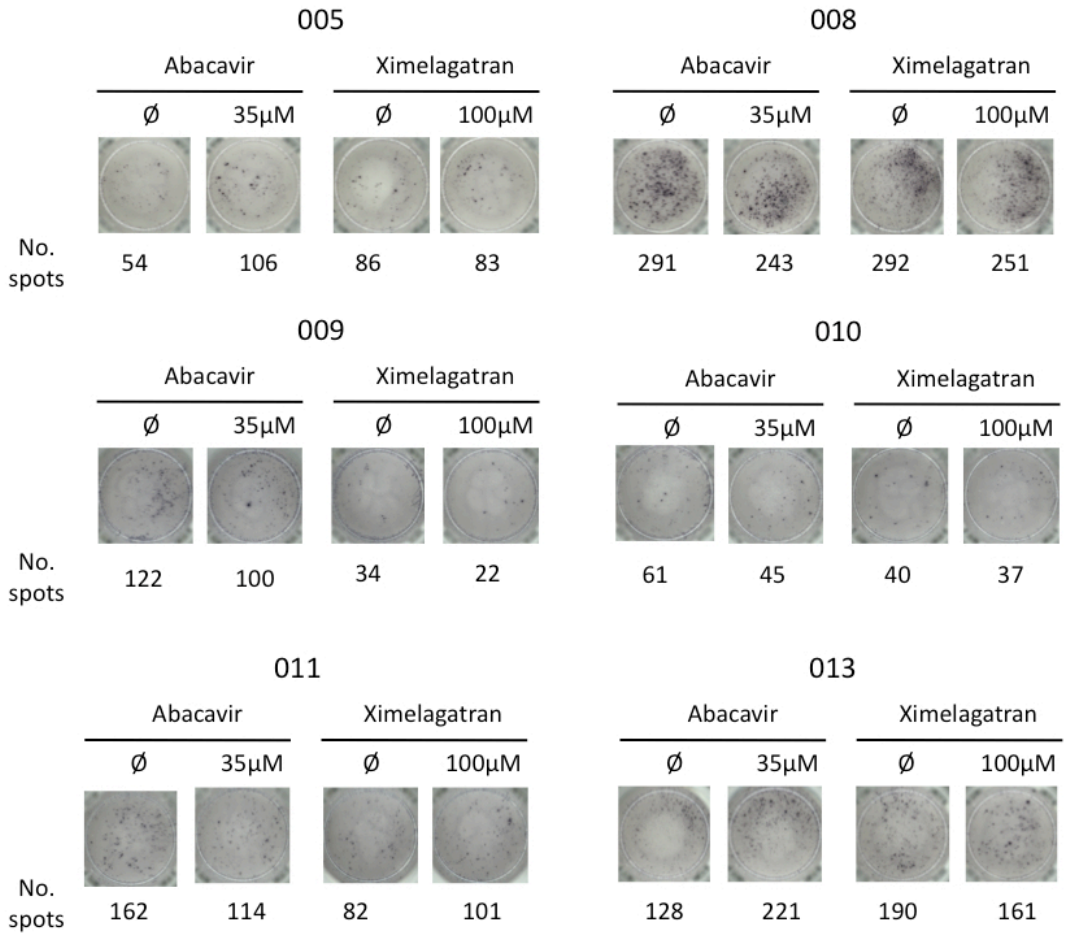

Figure 3.9 Drug-specific secretion of interferon-γ from T-cells following 14-day enrichment period. HLA-B*57:01 negative, HLA-DRB*07:01 positive volunteers. Following 14-day incubation with either abacavir or ximelagatran T-cells were harvested and restimulated with the antigen of interest. Well images are shown with spot counts assigned by AID Software.

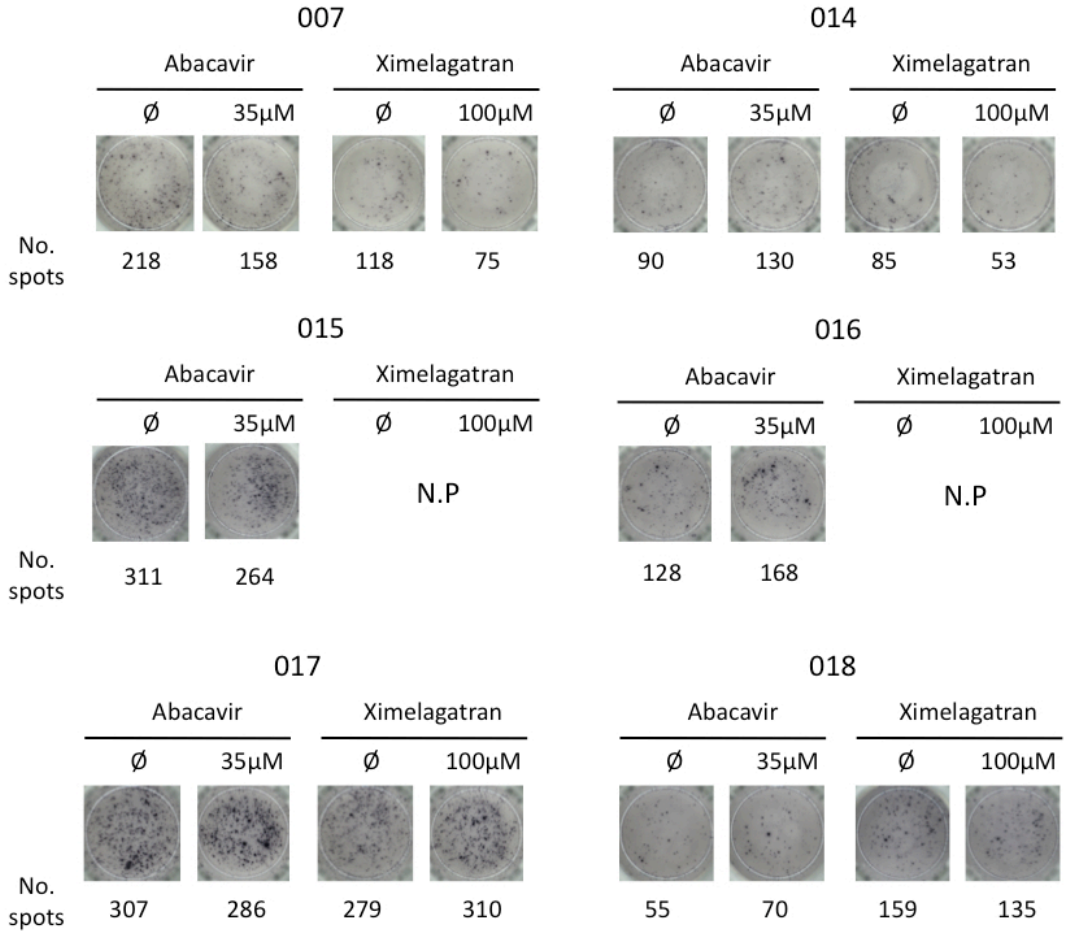

Figure 3.10 Drug-specific secretion of interferon-γ from T-cells following 14-day enrichment period. HLA-B*57:01 negative, HLA-DRB*07:01 positive volunteers. Following 14-day incubation with either abacavir or ximelagatran T-cells were harvested and restimulated with the antigen of interest. Well images are shown with spot counts assigned by AID Software. N.P = not performed.

3.4.2 Gene expression analysis

3.4.2.1 RNA integrity

The Agilent Bioanlayser was used in order to determine the quality of RNA and whether any degradation had occurred. The RIN is a score of between 1 and 10 with 10 being intact RNA and 0 being completely degraded. Some degradation was apparent in the samples analysed (Table 3.5). Though the RNA was not of particularly good quality, both the NanoString and TaqMan platforms are quite tolerant of RNA degradation and given the time taken to recruit all of the volunteers it was decided to continue with the analysis.

Table 3.5 Assessment of RNA yield and quality

Sample	RNA yield (ng)	Mean RIN (±SD)
004	3618 ± 451	3.2 ± 1.2
019	3374 ± 855	2.8 ± 0.9
020	3358 ± 879	3.8 ± 1.6
021	2468 ± 566	2.6 ± 0.8
022	3030 ± 757	4.6 ± 0.9
023	2803 ± 1174	3.0 ± 0.6

3.4.2.2 NanoString

The expression of 46 genes was analysed in RNA isolated from the PBMCs of six volunteers (3 x HLA-B*57:01 positive, 3 x HLA-B*57:01 negative). Due to the number of samples each sample could only be run once. Gene counts were therefore averaged and the average fold change was calculated. Tables detailing all of the gene counts and fold changes can be found in the Appendix. The 24h timepoint will be discussed here. A gene count of less than 20 was considered 'not expressed' and was therefore not included in the fold change analysis.

Genes that were upregulated by greater than 2-fold following 24h abacavir treatment included; CCL3 (both groups), CXCR3 (HLA-B*57:01 positive only), IL1R1 (HLA-B*57:01 positive only), IL-6 (both groups) and IL8 (both groups, although only >2 fold in HLA-B*57:01)(Figure 3.11). No genes were down regulated by greater than 2-fold following abacavir treatment. The considerable variation between individuals however meant that none of these differences were statistically significant (Figure 3.11).

Following 24h flucloxacillin treatment the following genes were affected by greater than 2-fold; IL-6 (upregulated in HLA-B*57:01 negative), CCL3 (HLA-B*57:01 positive = -3.3 fold; HLA-B*57:01 negative = 2.3 fold), CCL7 (HLA-B*57:01 positive only) and CCR1 (HLA-B*57:01 positive only).

Tetanus treatment was associated with the upregulation of the following genes; CCR5 (HLA-B*57:01 positive only), IL4R (HLA-B*57:01 positive only), IL6 (both groups), CCL7 (HLA-B*57:01 negative only), CCL4 (HLA-B*57:01 negative only), and CCL3 (HLA-B*57:01 negative only). No genes were down regulated following 24h tetanus treatment.

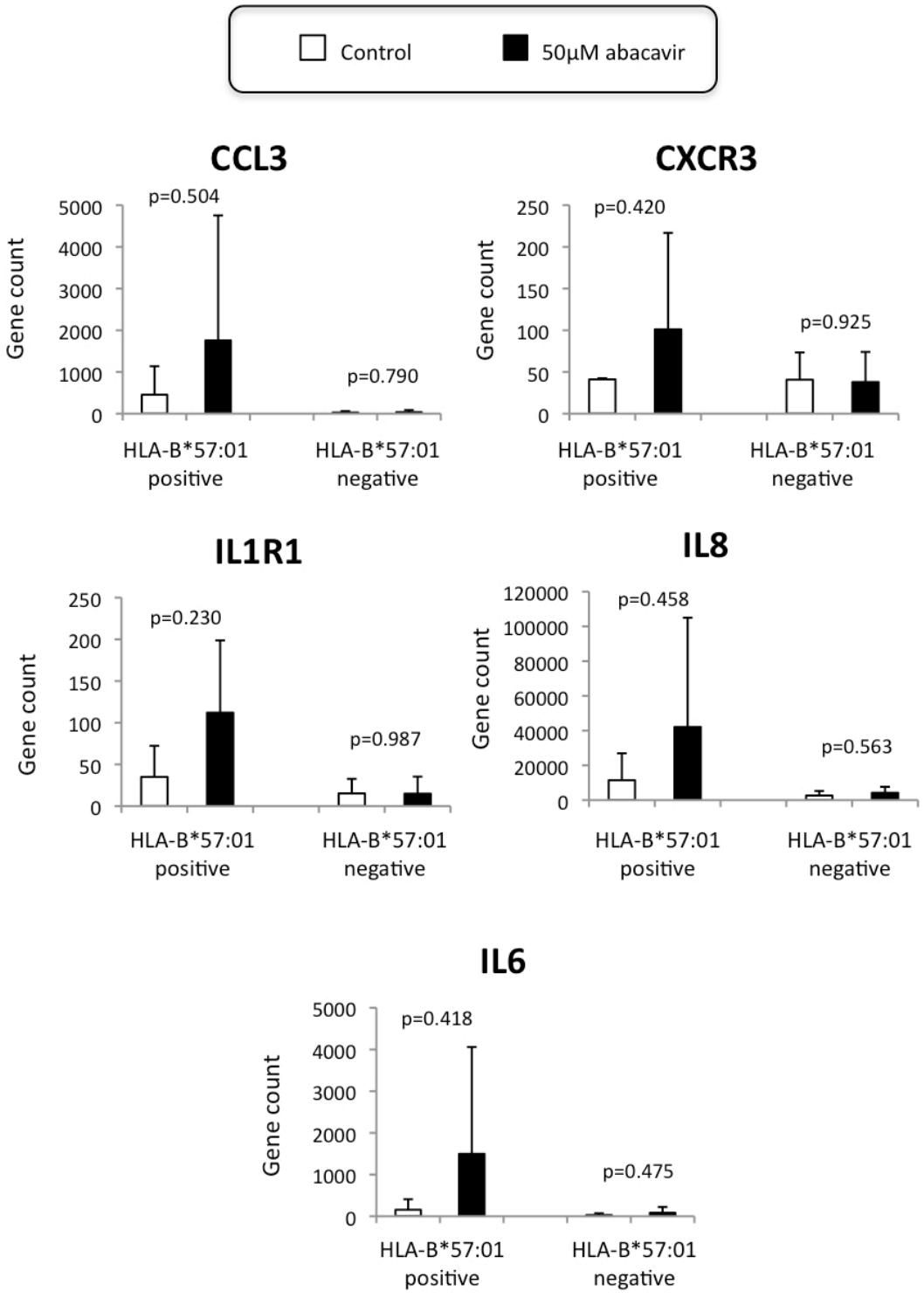

Figure 3.11 Changes in gene expression associated with abacavir treatment as determined by the NanoString platform. PBMCs from HLA-B*57:01 positive or negative volunteers were cultured with abacavir (50μM) for 24h. RNA was subsequently isolated and the expression of a panel of 46 genes was determined. Data were normalised to spiked-in controls and the housekeeping genes GUSB, HPRT and RPL19. Data represent mean + SD from three individuals. Statistical significance was determined by t-test.

3.4.2.3 TaqMan real-time PCR

Based on the NanoString data 5 genes were selected for further analysis: CCL3, CXCR3, IL-1R1, IL-6 and IL-8. Only the 24-hour timepoint was investigated. RNA was reverse transcribed and the resulting cDNA was incubated with TaqMan primers and probes against each of the selected targets. C_t values were determined and fold changes calculated. Considerable inter-individual variation was observed. In some instances abacavir (Figure 3.12) and flucloxacillin (Figure 3.13) caused both up-regulation and down-regulation of genes depending on the individual. None of the changes were statistically significant.

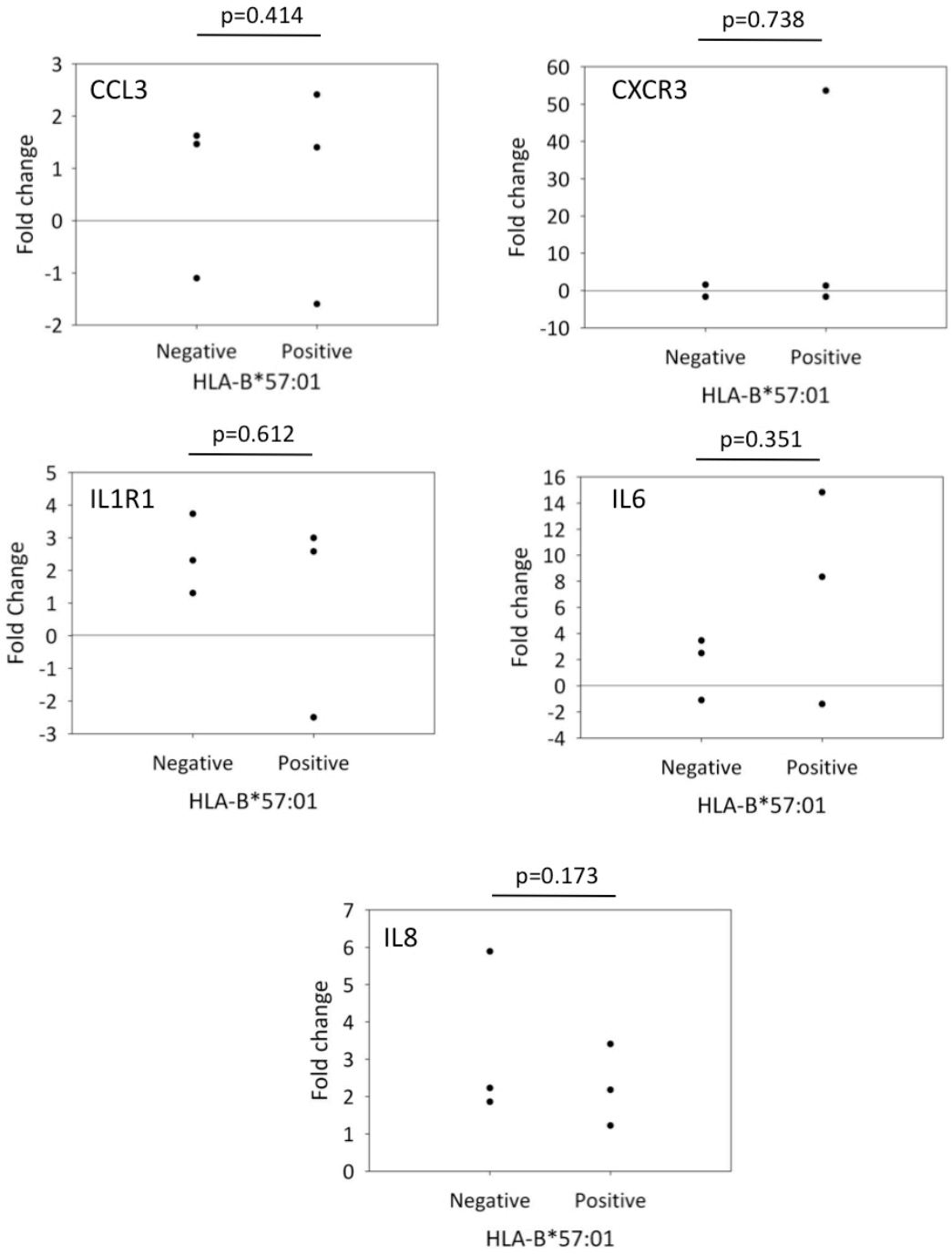

Figure 3.12 TaqMan analysis of gene expression following abacavir treatment. PBMCs from 3 HLA-B*57:01 positive and 3 HLA-B*57:01 negative volunteers were cultured with abacavir (50μM) for 24h. Data represent fold change compared to untreated samples. Data were normalised to the housekeeping gene GUSB. Statistical significance was determined by t-test.

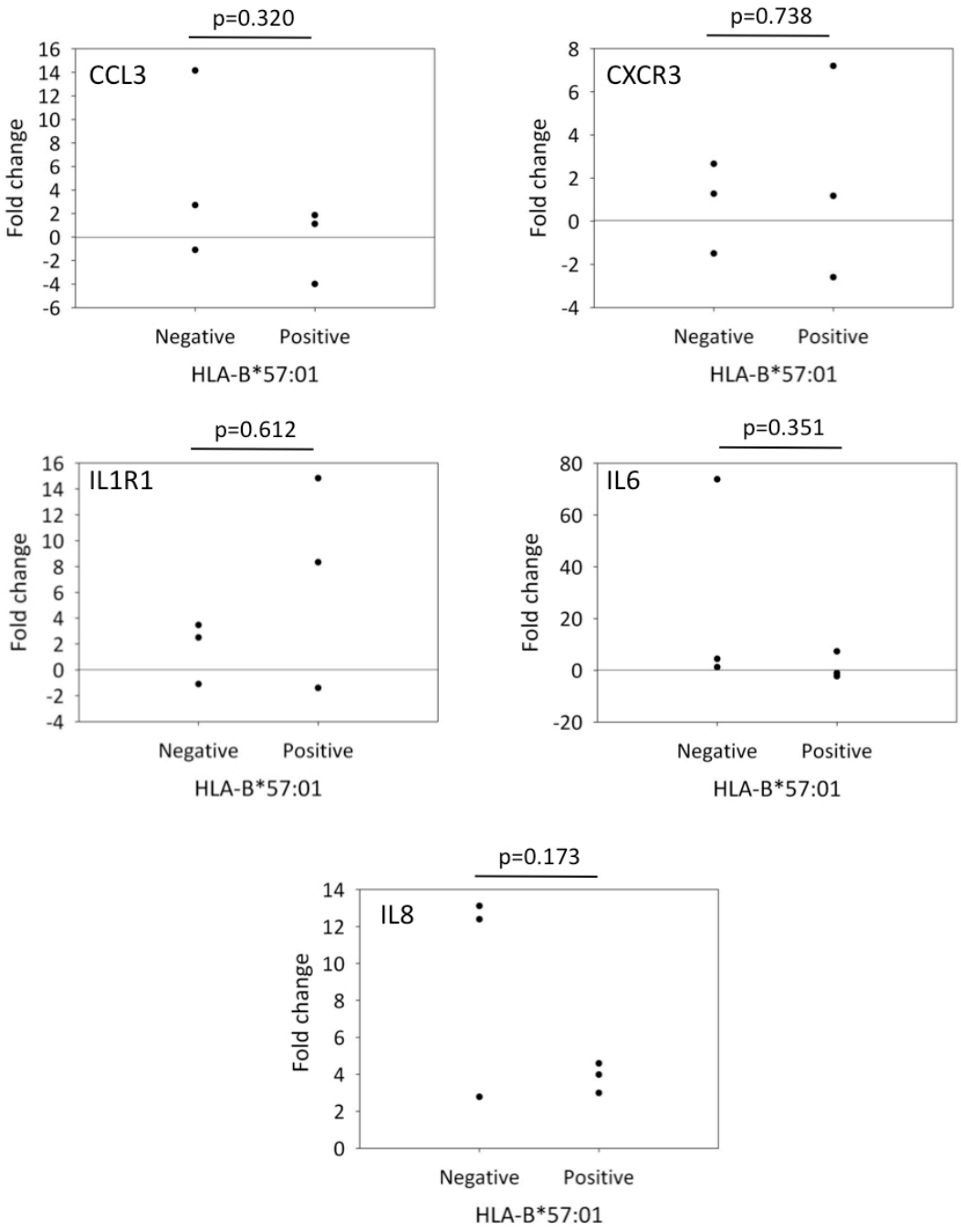

Figure 3.13 TaqMan analysis of gene expression following flucloxacillin treatment. PBMCs from 3 HLA-B*57:01 positive and 3 HLA-B*57:01 negative volunteers were cultured with flucloxacillin (1mM) for 24h. Data represent fold change compared to untreated samples. Data were normalised to the housekeeping gene GUSB. Statistical significance was determined by t-test.

3.4.3 T-cell cloning

3.4.3.1 Generation of abacavir-specific T-cell clones

PBMC from six volunteers (3 HLA-B*57:01 positive, 3 HLA-B*57:01 negative; Table 3.6) were cultured with abacavir for 14 days. Drug-enriched $CD4^+$ and $CD8^+$ T-cells were subsequently isolated, serially diluted and stimulated with allogenic irradiated PBMC, IL-2 and PHA to generate T-cell clones. Seventy-four abacavir-responsive $CD8^+$ clones were generated from 3 volunteers expressing HLA-B*57:01 (Table 3.3; Figure 3.14: 0, 9556±9131cpm; abacavir [50μM], 33409±26548cpm; $p<0.0001$). Abacavir stimulated drug-specific T-cell clones to proliferate and secrete interferon-γ over a range of concentrations of abacavir (Figure 3.15 A and B). In contrast, when over 400 $CD4^+$ clones were incubated with EBV-transformed B-cells and abacavir, specific reactivity was not detected (data not shown).

One hundred and ninety three $CD4^+$ and/or $CD8^+$ clones were generated from 3 HLA-B*57:01 negative volunteers and specific activity with abacavir tested. Abacavir-specific responses were not observed.

Table 3.6 Volunteer characteristics and generation of abacavir-specific T-cell clones. A lymphocyte transformation test (LTT) was performed in order to rule out any prior sensitivity to the drug. A stimulation index of > 2 was considered a positive result. Seventy-four $CD8^+$ T-cell clones were generated by serial dilution in total and used in subsequent mechanistic studies.

						ABC-specific clones	
Volunteer	**HLA alleles**	**Age**	**Sex**	**LTT**	**No. clones tested**	**$CD4^+$**	**$CD8^+$**
019	A*01:01/A*02:01 B*08:01/B*57:01 C*06:02/C*07:01 DRB1*03:01/DRB1*03:01 DQB1*02:01/DQB1*02:01 DQA1*05:01/DQA1*05:01	38	M	-	208	0	5
024	A*01:01/A*01:01 B*49:01/B*57:01 C*06:02/C*07:01 DRB1*16:01/DRB1*07:01 DQB1*03:03/DQB1*05:02 DQA1*01:02/DQA1*02:01	26	F	-	276	0	67
025	A*03:01/A*24:02 B*14:02/B*57:01 C*06:02/C*08:02 DRB1*13:02/DRB1*07:01 DQB1*03:03/DQB1*06:09 DQA1*01:02/DQA1*02:01	27	M	-	314	0	2
026	A*02:01/A*11:01 B*15:01/B*51:01 C*03:04/C*15:02 DRB1*01:01/DRB1*04:01 DQB1*03:02/DQB1*05:01 DQA1*01:01/DQA1*03:01	26	M	-	96	0	0
027	A*02:01/A*03:01 B*14:02/B*18:03 C*07:01/C*08:02 DRB1*13:02/DRB1*07:01 DQB1*02:01/DQB1*06:04 DQA1*01:02/DQA1*02:01	24	F	-	72	0	0
028	A*03:01/A*31:01 B*07:02/B*08:01 C*07:01/C*07:02 DRB1*03:01/DRB1*04:04 DQB1*02:01/DQB1*03:02 DQA1*03:01/DQA1*05:01	31	M	-	24	0	0

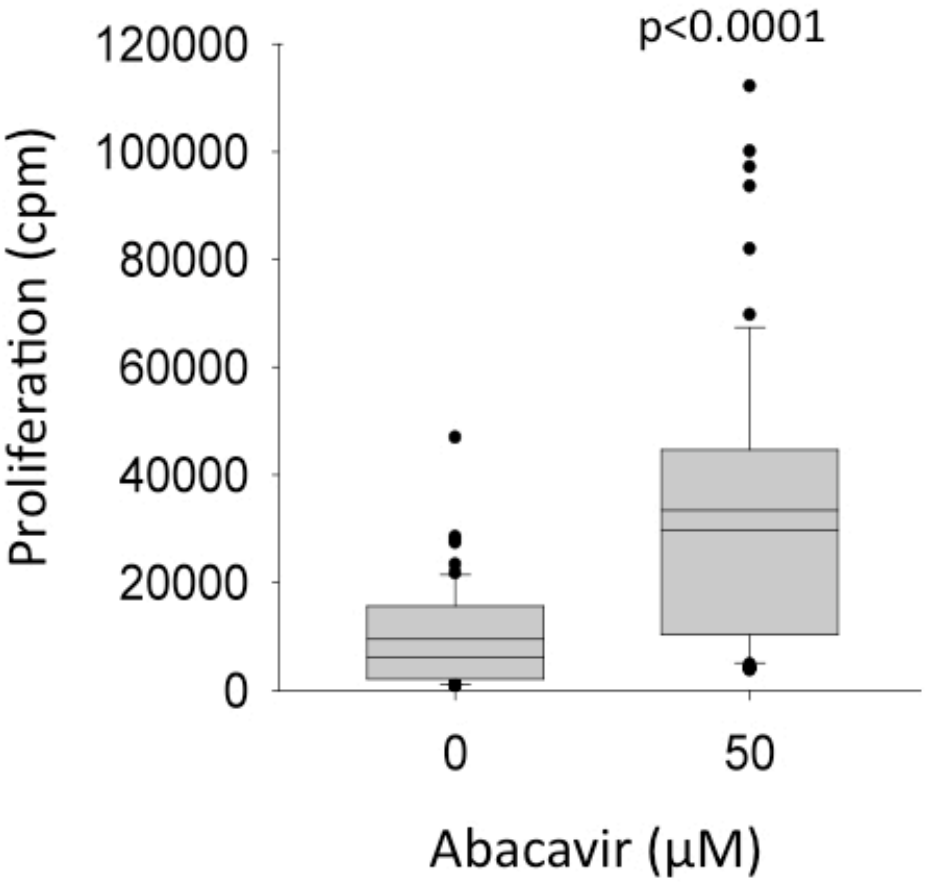

Figure 3.14 Proliferation of 74 abacavir-responsive clones from three HLA-B*57:01 positive volunteers stimulated with EBV-transformed B-cells in the presence or absence of abacavir (50µM)(p<0.0001, Mann-Whitney test).

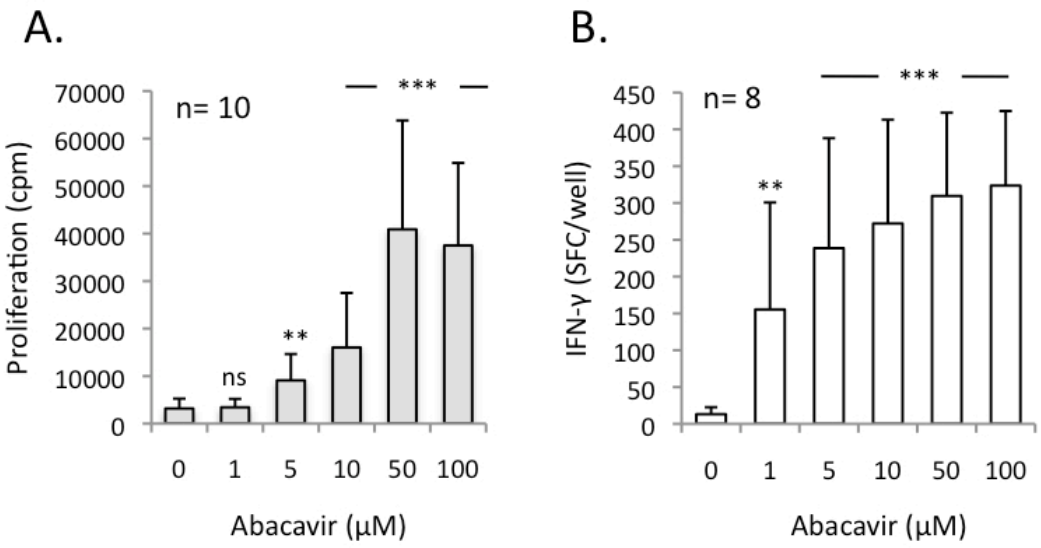

Figure 3.15 Abacavir-specific proliferation (A) and interferon-γ secretion (B) of T-cell clones isolated from HLA-B*57:01 positive drug-naïve volunteers. Data represent mean and standard deviation of the indicated number of clones. (ns not significant, **p<0.01, ***p<0.001; control vs. treated; Mann-Whitney test) SFC= spot forming cell.

3.4.3.2 T-cell receptor Vβ expression

CD and Vβ phenotyping was performed with a panel of over 30 antibodies using a BD FACSCanto II flow cytometer. The T-cell receptor Vβ repertoire was heterogeneous indicating that there is no shared TCR-Vβ useage (Figure 3.16). All clones tested also stained positive for CD8 confirming the expected phenotype.

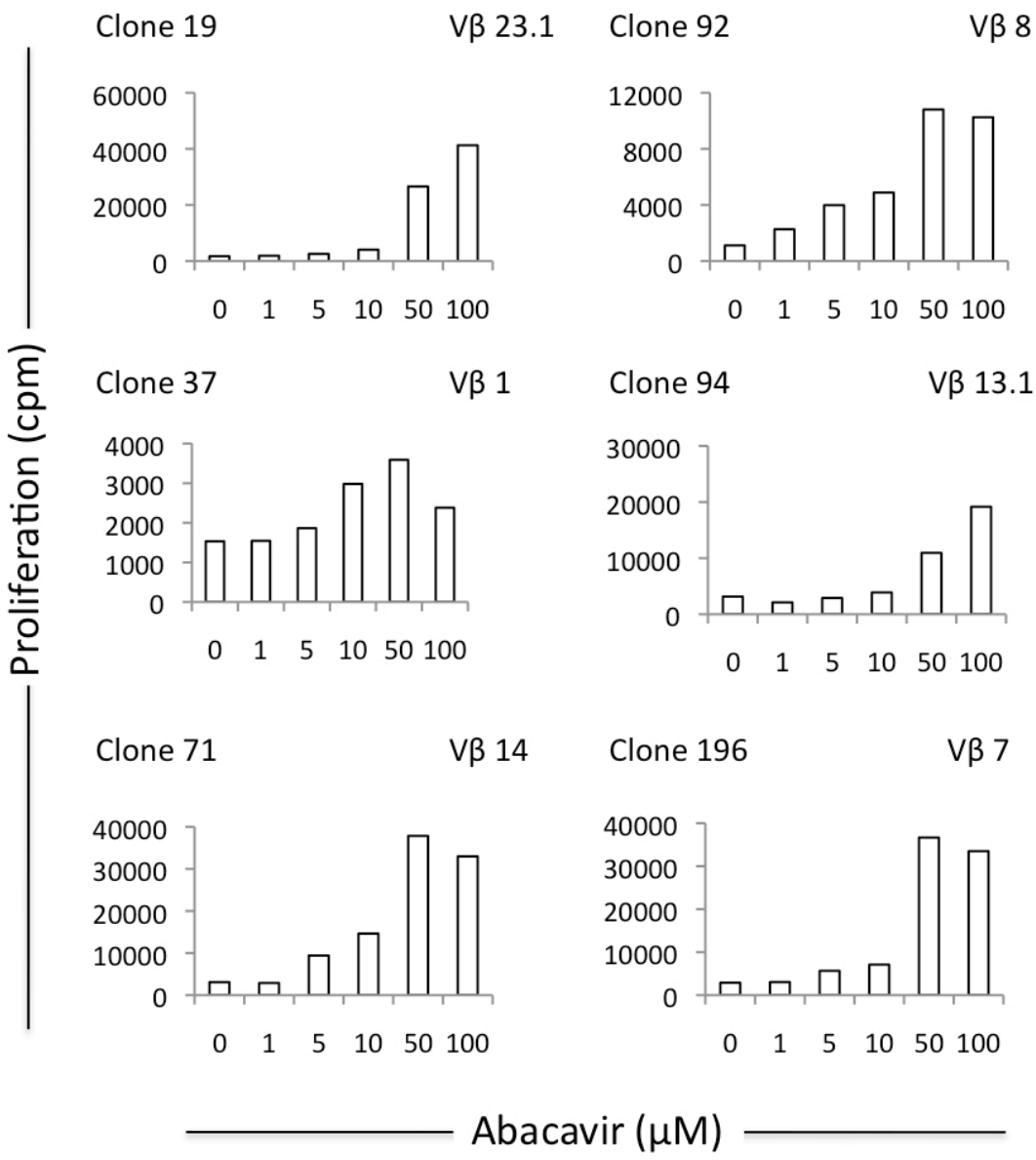

Figure 3.16 TCR Vβ expression is polyclonal. Representative figures of six T-cell clones, with varied TCR Vβ expression isolated from volunteer 024. Abacavir-specific proliferation as determined by [^{3}H]-thymidine incorporation is also displayed.

3.4.3.3 Secretion of cytokines and cytotoxic molecules

The abacavir-specific T-cell proliferative response was associated with high levels of IFN-γ, IL-13 and TNF-α secretion (Table 3.7 [Luminex analysis]; Figure 3.17 [ELISpot]). In contrast, cytokines IL-4, IL-5, IL-9 and IL-10 were secreted at lower levels and only by a limited number of clones. T-cell activation was

associated with the release of cytolytic molecules including perforin, granzyme B, and FasL (Figure 3.18 and 3.19). Clones were broadly divided into 3 categories: (1) clones secreting effector molecules and displaying increased FAS ligand expression (e.g., clones 12, 59 & 71); (2) clones primarily secreting effector molecules (e.g., clone 13); and (3) clones primarily displaying increased FAS ligand expression (e.g., clone 19).

Table 3.7 Luminex analysis of secreted cytokines and chemokines from 10 abacavir-specific T-cell clones

		Cytokine/Chemokine (pg/ml)							
Clone ID	Proliferation (Max SI)	IL-2	IL-4	IL-5	IL-10	IL-13	GM-CSF	IFN-γ	TNF-α
12	23	16	nd	nd	nd	43	36	86	21
13	7	nd	nd	nd	nd	20	nd	2	22
19	39	72	nd	nd	nd	71	12	75	188
37	6	nd	nd	8	3	4009	nd	3	11
59	13	nd	nd	8	nd	2857	nd	nd	37
71	12	72	nd	2	nd	1454	102	286	560
105	9	46	nd	36	2	7089	146	108	351
116	21	nd	nd	nd	nd	336	nd	nd	20
160	11	3500	7	182	nd	>10,000	>10,000	8002	>10,000
168	41	928	8	412	nd	>10,000	1345	1649	2994

Data represents mean of duplicate cultures with cytokine levels (less than 5pg/ml IL-4, IL-9, IL-10, IL-12, GM-CSF, IFN-γ, TNF-α; less than 20pg/ml less than 50pg/ml IL-2, IL-5, IL-13) in drug-free wells subtracted. nd= not detectable. IL-9 and IL-12 were not detectable in any sample and so are not shown in the table. Th1 cytokines are highlighted in blue and Th2 cytokines are shown in red.

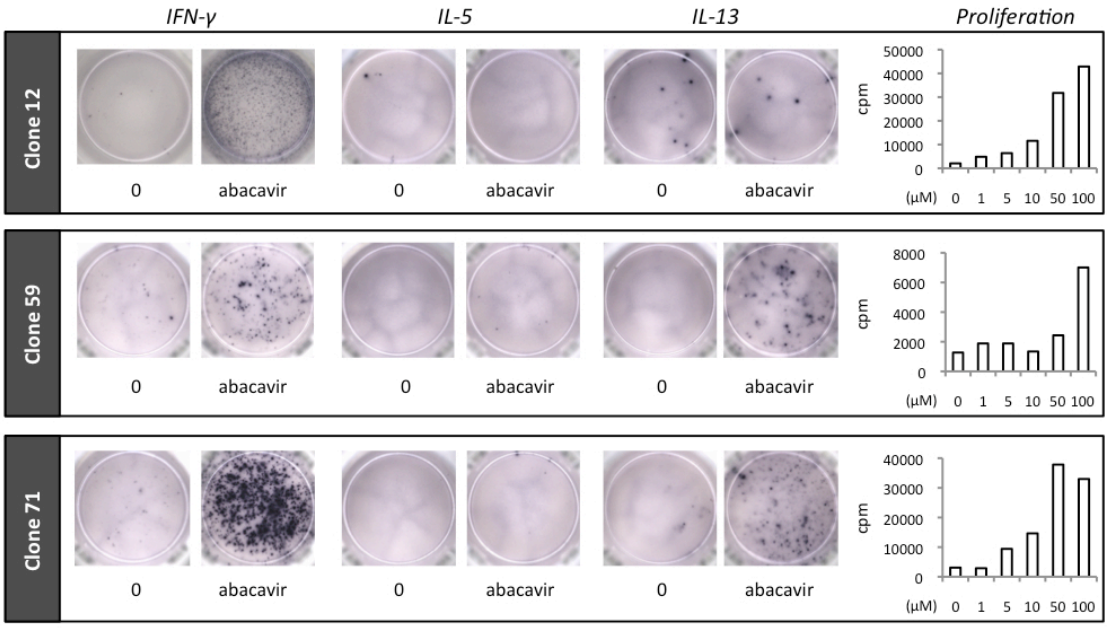

Figure 3.17 Secretion of interferon-γ, interleukin-5 and interleukin-13 from abacavir-specific T-cell clones as determined by ELISpot assay. Proliferation was measured by [^{3}H]-thymidine incorporation. Clones were isolated from volunteer 024.

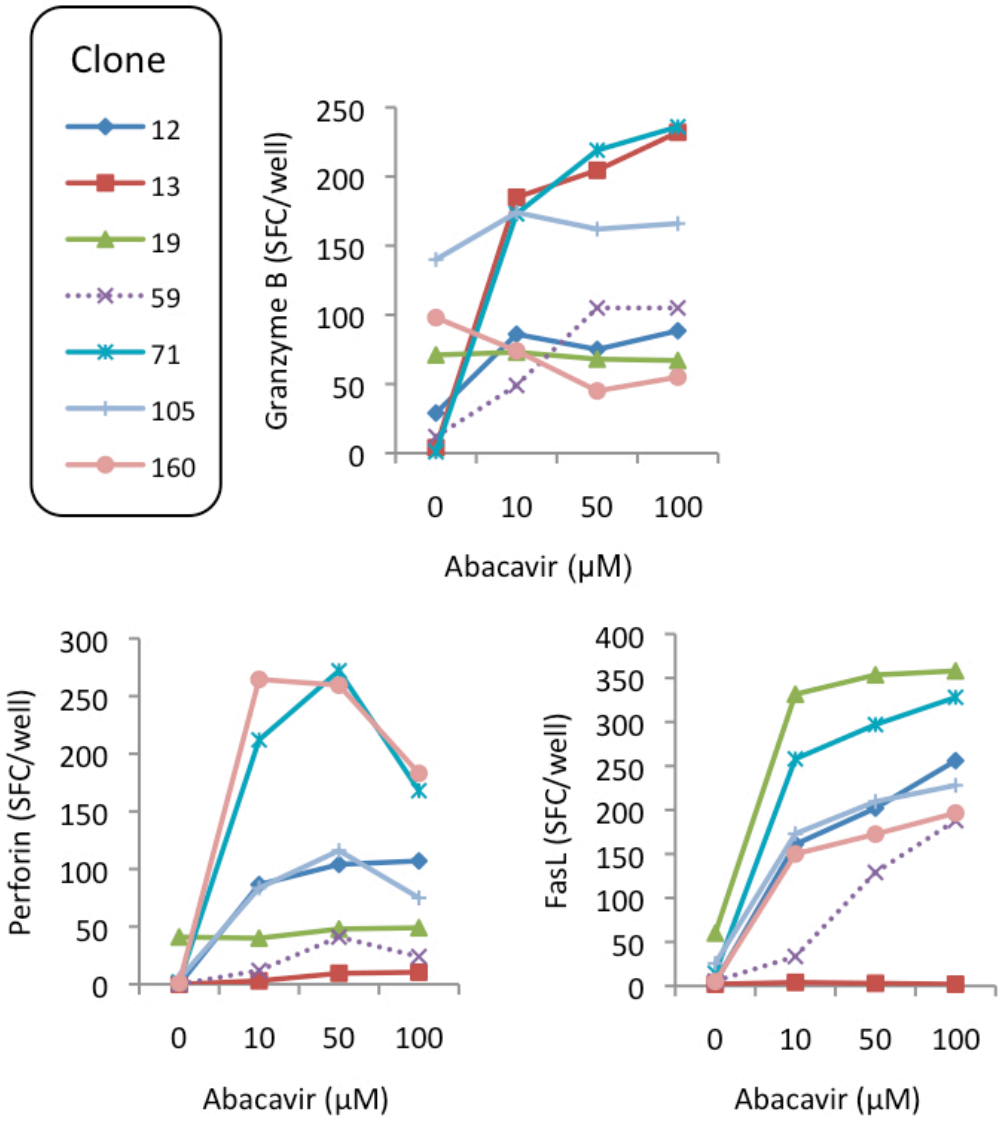

Figure 3.18 Abacavir-specific secretion of effector molecules (Granzyme B, perforin and Fas ligand) from seven abacavir-specific T-cell clones. Determined by ELISpot assay. SFC= spot forming cell. Clones were isolated from volunteer 024.

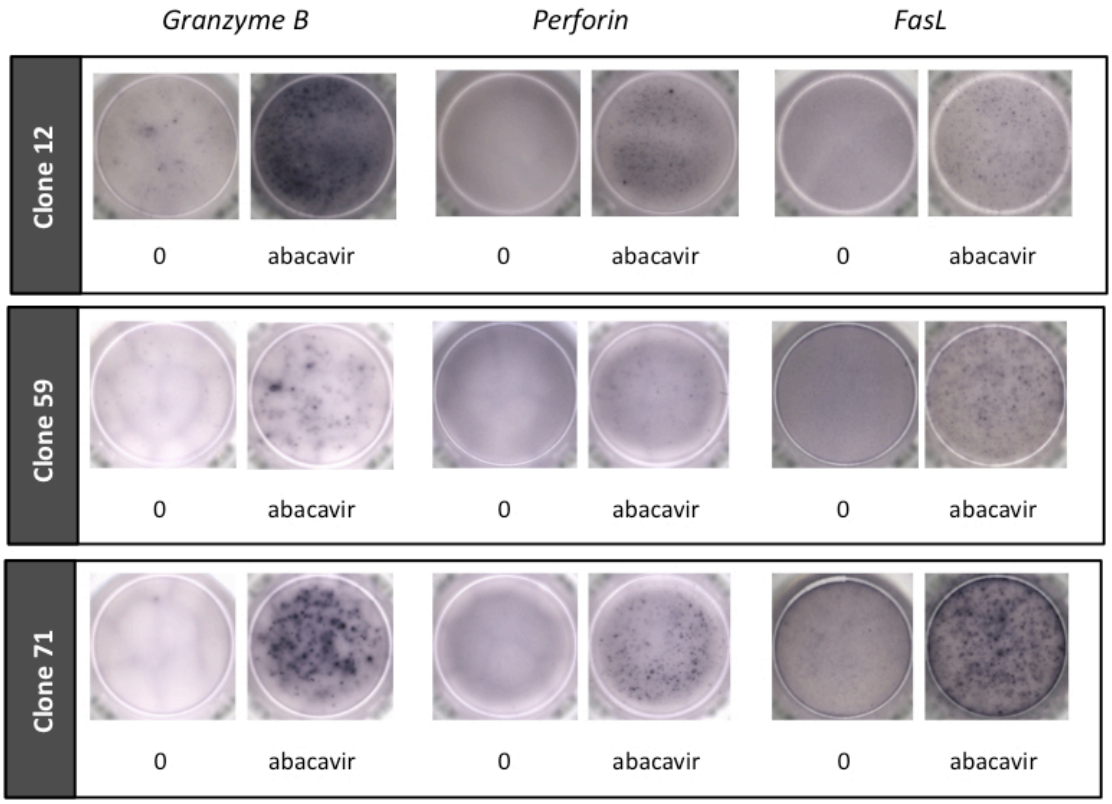

Figure 3.19 Abacavir-specific secretion of cytotoxic molecules granzyme B, perforin and Fas ligand from T-cell clones. Clones were incubated with 50μM abacavir and autologous antigen-presenting cells in antibody coated filter plates. ELISpots were developed after 48h. Clones were isolated from volunteer 024.

3.5 Discussion

T-cells are often implicated in the development of immunological reactions to drugs. The proliferation of drug-specific T-cells *ex vivo* is commonly used to diagnose drug hypersensitivity reactions and to determine the causative agent where multiple therapies are administered concurrently.

Recently, abacavir-specific T-cells have been identified from drug-naïve volunteers expressing HLA-B*57:01, the HLA allele associated with susceptibility to abacavir hypersensitivity. This was dependent on a 14-day pre-

enrichment period in the presence of IL-2 (Chessman et al., 2008). Including a pre-enrichment step has previously been found to increase the sensitivity of the standard LTT assay when applied to SMX hypersensitive patients (Lavergne et al., 2010). It was also determined that this increase in sensitivity was not due to a primary stimulation, as drug-specific T-cells were not detected in PBMCs of tolerant or naïve volunteers after following this protocol (Lavergne et al., 2010). It would therefore seem that this pre-enrichment step results in an expansion of the drug-specific T-cells already present in the peripheral blood.

In this investigation, very limited proliferative responses to abacavir were observed in lymphocytes from 2/6 HLA-B*57:01 positive volunteers. This is in contrast to Chessman et al. (2008), who were able to detect drug-specific responses in all volunteers expressing the risk allele. In that study the T-cell response was quantified in terms of intracellular IFN-γ staining. Incorporation of [^{3}H]-thymidine was chosen as an endpoint for this investigation as it commonly used to detect drug-specific T-cells in the peripheral blood of hypersensitive patients (El-Ghaiesh et al., 2011, Castrejon et al., 2010) and is an accurate measurement of cellular proliferation. However a disadvantage of [^{3}H]-thymidine incorporation as a readout is that it is not able to differentiate between many cells proliferating slightly and a few cells undergoing many divisions. Each measurement is based on the entire well. Flow cytometry techniques however can measure the response of single cells. Carboxyfluorescin diacetate succinimidyl ester (CFSE) is a fluorescent dye, which is split between daughter cells during each cell division. The subsequent halving of fluorescent intensity can be quantified (Lyons, 1999) (Fulcher and Wong, 1999). A

particular advantage of this technique is that cell populations e.g $CD4^+/CD8^+$ can be analysed separately (Hanafusa et al., 2012).

The cytokine readout employed in this investigation (ELISpot assay) did not detect any abacavir-specific increase in IFN-γ secretion from lymphocytes expressing HLA-B*57:01, following the 14-day pre-enrichment. Though the ELISpot has a lower limit of detection (approximately 10 cytokine secreting cells per 1 million PBMCs), intracellular staining may be more sensitive in cells producing low amounts of cytokine (Letsch and Scheibenbogen, 2003). High levels of non-specific IFN-γ secretion were detected, possibly due to cells having been incubated with the drug in the presence of IL-2 for 14 days. Though cells were washed prior to performing the ELISpot, abacavir may induce danger signalling (Martin et al., 2007) and so therefore the cells could have been in an activated state already. The addition of cytokines such as IL-15 have recently been shown to aid the detection of drug/antigen-specific T-cells (Zawodniak et al., 2010), however the day on which they are added can influence the non-specific activation of T-cells (Munier et al., 2009).

Much less evidence for the involvement of T-cells in the ALT elevations associated with ximelagatran treatment exists. Positive LTTs have been observed in 2/21 patients with ALTs > 4 x upper limit of normal (maximum SI 3.2 and 2.0 to melagatran and ximelagatran respectively)(Kindmark et al., 2008). In this investigation a borderline positive proliferative response was seen in 1/12 HLA-DRB*07:01 positive volunteers (SI= 1.9). No responses were detected by IFN-γ ELISpot. The odds ratio for HLA-DRB*07:01 is just 4.4 compared to 117 for abacavir indicating that there are likely to be other factors

accounting for individual susceptibility (Kindmark et al., 2008, Mallal et al., 2008).

Drug concentrations selected for the study were based on the maximum non-toxic stimulatory concentration. For abacavir this was taken from a previous study (10μg/ml is equal to approximately 35μM) (Chessman et al., 2008). For ximelagatran few long-term *in vitro* studies have been performed with primary immune cells. The monocyte-like cell line THP-1 showed some decrease in viability following 24h treatment with 100μM ximelagatran (Edling et al., 2008). Hepatocytes however are more tolerant to ximelagatran and a loss in viability is not observed until concentrations exceeding 200μM (Kenne et al., 2008). A dose of 100μM was therefore selected. Due to the cell numbers required melagatran could not be included.

Given that the data of Chessman et al. could not be replicated, a second approach was adopted, namely the investigation of gene expression. Given that both abacavir hypersensitivity and flucloxacillin-induced liver injury are associated with carriage of HLA-B*57:01 the two drugs could be investigated in the same samples. In this study, the integrity of the isolated RNA was not ideal, however recruiting volunteers again, repeating the incubations and then re-extracting the RNA was not feasible. When performing microarray analysis high quality RNA is required, generally a RIN >7 is advised (Thompson et al., 2007). Samples were frozen until all of the volunteers had been recruited. It may have been better to have isolated the RNA rapidly and then stored that until all of the samples had been collected. One of the benefits of using the NanoString

platform however is that it is relatively tolerant to RNA degradation. A recent study directly compared tissue samples that had been either flash frozen (RIN= 4.6-9.8) or formalin-fixed and paraffin-embedded (FFPE)(RIN= 1.5-2.5). The authors found that there was a good correlation between the gene expression data obtained from the two sets of samples (Reis et al., 2011). For this reason it was decided to continue with the study.

Following 24 hour treatment with abacavir a number of genes showed an increase in expression that may have been promising to investigate further (CCL3, CXCR3, IL1-R1, IL-6 and IL-8). Though these changes were not statistically significant, a confirmatory real-time PCR study was designed. Technical replicates could be included in the TaqMan setup and it was hoped that this would reduce the variation observed. This was not found to be the case. It would therefore seem that there is significant inter-individual variation in the expression of cytokines and chemokines. This may be a factor in the development of hypersensitivity reactions, however it is unclear why this occurs. Zidek et al. (2007) found considerable variation in baseline levels of CCL3 in a study investigating the effects of tenofovir treatment. Despite this the authors were able to obtain consistent fold-change increases following drug treatment. A larger sample size may be required in order to improve the data.

Due to the difficulties in detecting drug-specific T-cells by the previous methods T-cell cloning was attempted. T-cell clones are monoclonal cell populations derived from a single T-cell. $CD4^+$ and $CD8^+$ T-cells were separated prior to serial dilution, as $CD8^+$ T-cell clones are particularly difficult to isolate (Wu et

al., 2007). Abacavir-specific T-cell clones with a clear Th1 cytokine profile were isolated from all three HLA-B*57:01 positive volunteers included in this part of the study. The varying number of clones generated from each individual may relate to technical issues; in particular, the extensive handling needed to isolate and culture $CD8^+$ clones (Wu et al., 2007). However, as 45% of individuals who carry the HLA-B*57:01 allele do not develop hypersensitivity when exposed to abacavir (Mallal et al., 2008), it is also possible that the number of detectable clones is one factor that determines susceptibility. $CD4^+$ clones were generated in large numbers, but abacavir-specific responses were not detected, which is consistent with the class I HLA association.

Volunteers were all LTT negative indicating that they were not previously sensitised to abacavir, though given that drug-specific T-cell clones could be isolated from the three HLA-B*57:01 positive volunteers this may suggest that the frequency of drug-specific cells in the circulation is below the limits of detection of the LTT.

Vβ expression was investigated both to confirm the monoclonality of T-cell clones and to investigate TCR restriction. When investigating carbamazepine-induced SJS/TEN, Ko et al. (2011) found that in addition to HLA-B*15:02, patients experiencing SJS/TEN also shared a distinct TCRVβ-11 and TCRVβ-15 usage. The authors go as far as to suggest genotyping for this in the clinic. In this investigation the TCR-Vβ repertoire was heterogeneous as has previously been suggested (Chessman et al., 2008, Illing et al., 2012b, Illing et al., 2012a).

This would suggest that multiple epitopes are recognised by different T-cell clones and hence are immunogenic.

Cytotoxic T-cells are involved in different forms of immunological drug reaction (Kuechler et al., 2004). Following drug-induced T-cell receptor triggering, target cell killing might result from increased surface expression of FAS ligand and/or release of soluble mediators such as granzymes, granulysin and perforin. FAS ligand activates the apoptotic cascade through ligation of FAS receptors expressed on target cells. Perforin disrupts cell membranes, while granzymes and granulysin induce apoptosis, at least in part, through direct activation of caspases (granzymes, caspase 3 and 9; granulysin, caspase 7) (Thomas et al., 2001, Saini et al., 2011). The drug-specific release of Granzyme B from PBMCs can be used to identify patients with drug allergy (Zawodniak et al., 2010). The findings presented here demonstrate that clones release perforin, granzyme B and increase surface expression of FasL following activation with abacavir. Furthermore, it was possible to group the clones into 3 categories: (1) clones secreting effector molecules and displaying increased FAS ligand expression; (2) clones secreting effector molecules; and (3) clones displaying increased FAS ligand expression. These data support a recent report describing distinct granzyme-containing and FAS ligand-associated secretory lysosomes in human T-cells (Schmidt et al., 2011) and suggest that lysosomes are differentially expressed in clones and/or differentially activated in response to T-cell receptor signalling.

The isolation of abacavir-specific T-cell clones from drug-naïve HLA-B*57:01 positive volunteers provides an experimental tool to further investigate the role of HLA molecules in T-cell activation by drugs. The source of these cells remains unclear, as do the mechanisms by which they are activated. Drugs are thought to interact with T-cells by two key mechanisms; the hapten hypothesis and the p-i concept (discussed in 1.5). Further studies are required in order to investigate these mechanisms and to determine the specificity of the T-cell response.

Chapter Four

Development of an LC-MS/MS method for the identification and quantification of abacavir and metabolites in human liver cytosol and immune cell preparations

4.1 Introduction

Chemically reactive metabolites are often implicated in the toxicity of drugs. They can have direct toxic effects through binding to cellular macromolecules, which may lead to hapten formation.

The major metabolites formed *in vivo* following abacavir administration are a glucuronide and a carboxylic acid, accounting for 36% and 30% of the excreted dose respectively (Figure 4.1)(McDowell et al., 1999). The oxidation of abacavir to carboxylic acid is thought to proceed via a protein-reactive aldehyde metabolite (Walsh et al., 2002). As yet this has not been isolated either *in vitro* or *in vivo,* due to its apparent instability. Evidence for its formation is based on the observation of an oxime conjugate when methoxylamine is added to *in vitro* incubations of abacavir and human liver cytosol (Walsh et al., 2002).

The oxidation of abacavir is catalysed by alcohol dehydrogenase (ADH). Walsh *et al*. (2002) attributed this to the class I isoforms 1A1 and 1C2 in particular. Different reactions however were catalysed by each isoform (Figure 4.2). Incubation of abacavir with recombinant human ADH1A1 showed a very similar profile to whole cytosol, where multiple isomeric carboxylic acids were detected. Incubation of abacavir with ADH1C2 did not yield any carboxylic acids or indeed any more polar products. Instead a slightly less polar product was observed, which is believed to be an isomer of abacavir.

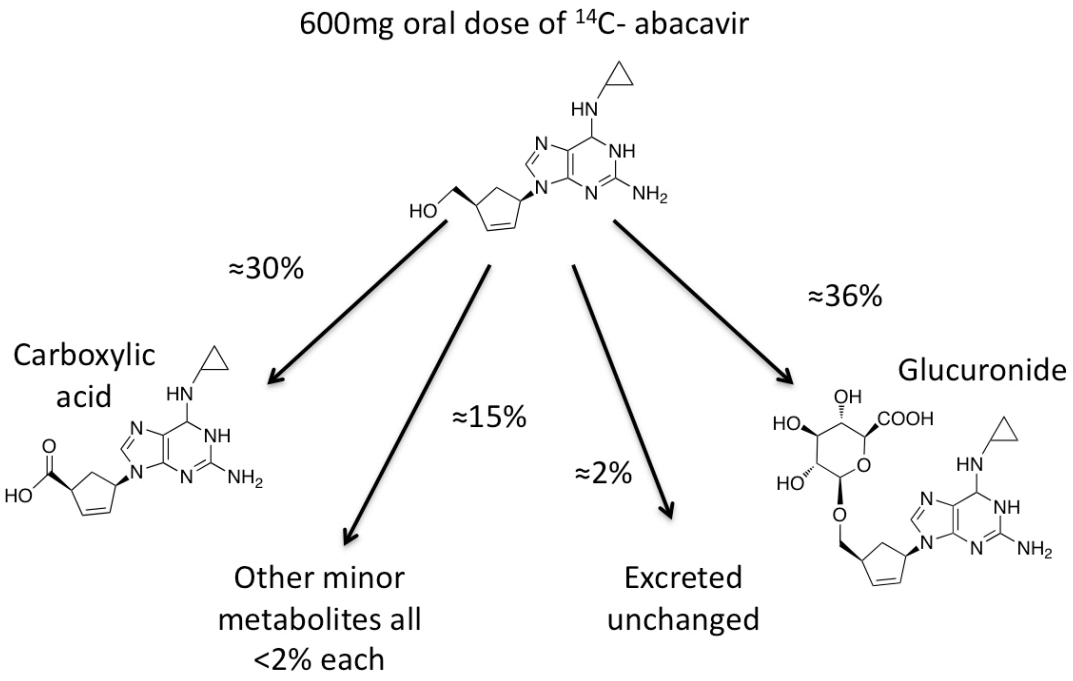

Figure 4.1 *In vivo* metabolism of abacavir results in the formation of two major metabolites, a carboxylic acid and a glucuronide (McDowell et al., 1999).

The adduction reactions of a synthetic aldehyde metabolite were recently characterised (Charneira et al., 2011). The mechanism of adduct formation was found to be dependent on the chosen nucleophile. Conjugation to nitrogen-containing nucleophiles (e.g valine) was found to occur via Schiff-base formation whereas conjugation to sulphur-containing nucleophiles (e.g cysteine) was by 1,4 addition (Charneira et al., 2011)(see 1.8.2 for mechanisms). *In vitro* incubation of the conjugated aldehyde with haemoglobin resulted in the formation of an N-terminal valine adduct (Charneira et al., 2011). These adducts were then used as standards to determine whether similar adducts were formed *in vivo*. When abacavir was administered to rats valine adducts could be detected in 2/6 (1 male, 1 female) animals (Charneira et al., 2012). Cysteine and lysine adducts were not detected. It is unclear however whether these metabolites are immunogenic or indeed formed in humans. Aldehydes are

however commonly associated with immunological reactions and in particular contact dermatitis due to their protein reactivity (O'Brien et al., 2005).

In order to investigate T-cell cross-reactivity a number of analogues of abacavir have been synthesised (Figure 4.3). These include compounds in which key functional groups are absent e.g dihydro abacavir and compounds with altered stereochemistry e.g abacavir enantiomers. The alternative enantiomer of abacavir (1*R*,4*S*) has the same chemical properties and chemical formula as 1*S*,4*R* abacavir but the stereochemistry of the cyclopentane ring is altered. It is pharmacologically inactive due to the stereoselectivity of adenosine phosphotransferase (Faletto et al., 1997). Dihydro abacavir lacks a double bond in the cyclopentane ring, which may be important for the binding of subsequent metabolites to proteins (Walsh et al., 2002). Introduction of a deuterium atom into a drug molecule can result in a slower rate of metabolism if cleavage of that bond is a rate limiting-step (Mutlib, 2008). This effect has been observed for a number of drugs and has in some instances been shown to reduce the toxicity associated with these compounds (Branchflower et al., 1984). It is important to understand how each of these changes to the abacavir molecule will impact upon metabolism, prior to using them in T-cell assays.

In this chapter the establishment and validation of an analytical method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the detection and quantification of abacavir and metabolites is presented. This methodology was then applied to directly investigate the metabolising capacity of antigen-presenting cells.

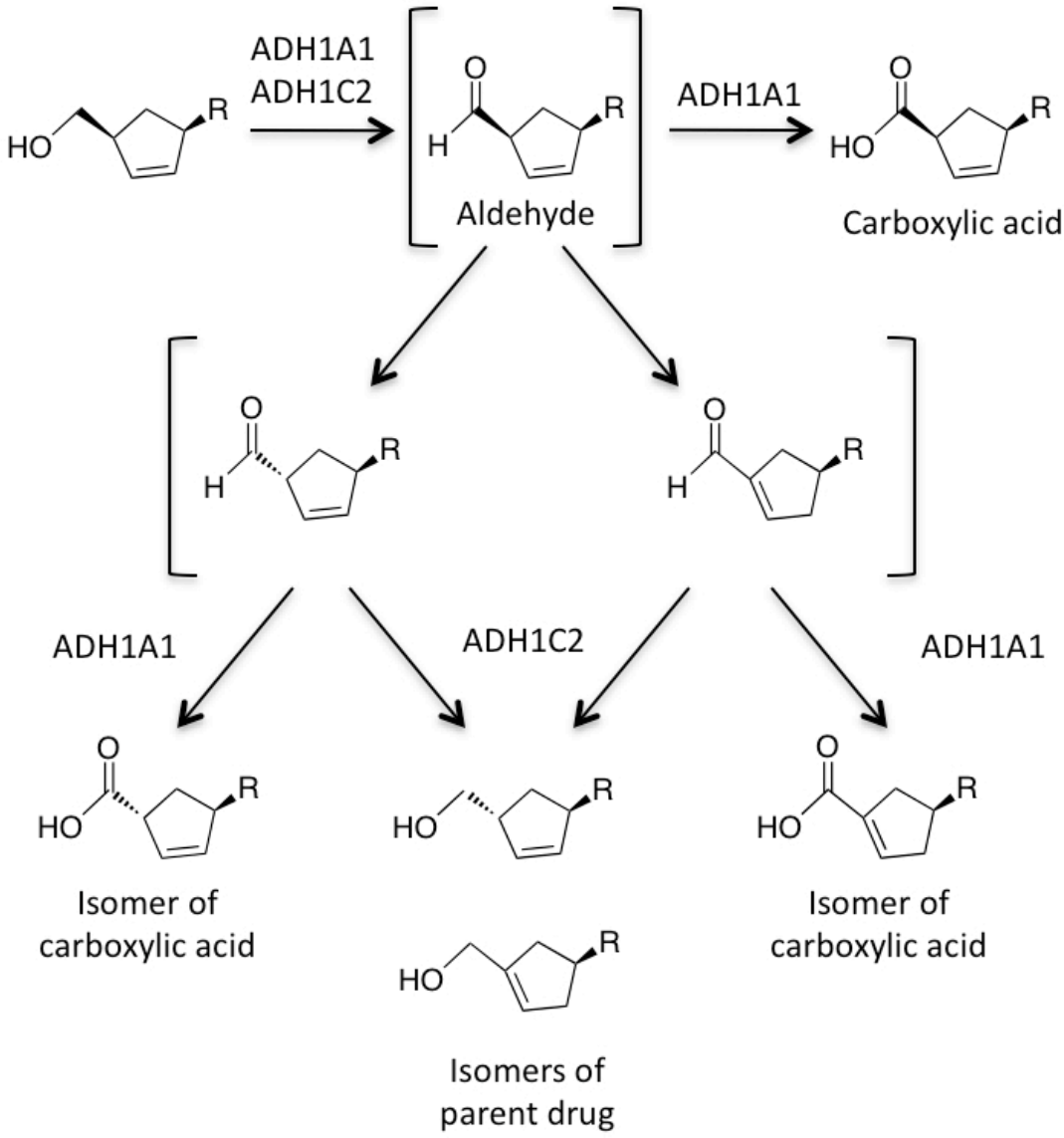

Figure 4.2 Proposed mechanisms of abacavir oxidation and rationale for multiple isomeric products observed *in vitro*. Adapted from (Walsh et al., 2002).

4.2 Aims

- Develop an LC-MS/MS method for the chromatographic separation and quantification of abacavir and metabolites
- Identify metabolites formed from abacavir analogues
- Determine the metabolising capactity of EBV-transformed B-cells

4.3 Materials and methods

4.3.1 Chemicals and reagents

Nicotinamide adenine dinucleotide (β-NAD), LC-MS grade water and acetonitrile were all bought from Fisher scientific, Loughborough, UK. MultiScreen Solvinert hydrophilic PTFE filter plates (0.45µm) were purchased from Merck Millipore, Watford, UK. RNeasy mini kit and RNase-free DNase kits were purchased from Qiagen, Crawley, UK. The RT^2 Profiler™ Drug metabolising enzyme PCR array was also purchased from Qiagen.

Abacavir sulfate and carboxylic acid were kind gifts from GlaxoSmithKline. Deuterated abacavir, dihydro abacavir and 1*R*,4*S* abacavir were synthesised by Emma Yang (Chemistry department, University of Liverpool). All other chemicals and reagents were obtained from Sigma Aldrich, Dorset, UK

4.3.2 LC-MS/MS conditions

Standards and samples were analysed on a 4000 QTRAP system (AB Sciex, Foster City, CA, USA). Chromatographic separation was performed on an Eclipse XBD C18 (2.1x150mm, 5µm) column (Agilent, Cheshire, UK) at 30°C. An Ultimate 3000 liquid chromatography system (Dionex, CA, USA) was interfaced to the mass spectrometer. Mobile phase A consisted of 10mM ammonium acetate pH 3.8 and mobile phase B consisted of acetonitrile. The compounds were eluted from the chromatographic column using a linear gradient (5-20% over 20 minutes) with a flow rate of 0.2 ml/min. The mass spectrometer was operated

in positive ion mode. Nitrogen was used as both the nebulising and drying gas. The source was operated at 500°C. The electrospray voltage was set to 5kV. Analyses were performed in MRM scanning mode. Compound-dependent parameters were optimised for each available standard and are shown in Table 4.1.

Table 4.1 Compound-dependent parameters

Compound	Precursor ion *m/z*	Product ion *m/z*	Declustering potential (Volts)	Collision energy (Volts)
Abacavir	287	191	66	27
Carboxylic acid	301	191	63	29
2-chloroadenosine	302	170	68	28

4.3.3 Abacavir metabolism in human liver cytosol

Reactions were performed in 50mM sodium pyrophosphate pH 8.8. Human liver cytosol (1mg/ml; 500µl) was incubated with abacavir (10µM) in the presence or absence of the co-factor NAD (7.5mM) at 37˚C for 1, 4 or 20 hours. Where used, 4-methylpyrazole (4-MP) was added at the beginning of the reaction at a final concentration of 0.6mM. Methoxylamine was used at a concentration of 10mM. To stop the reaction, samples were placed on ice for 5 minutes. Ten volumes of acetonitrile were added to a 100µl aliquot of the supernatant and 100µl internal standard (2-chloroadenosine; 2µM). Samples were vortexed and precipitated protein was removed by centrifugation (13,000g, 15min, 4˚C). The resulting supernatant was then transferred to a clean tube and evaporated to dryness. For mass spectrometric analysis the dried samples were reconstituted in mobile phase (100µl; 10mM ammonium acetate pH3.8 containing 5%

acetonitrile) and filtered through a 0.45μm filter plate. Data was processed using Analyst software. Relative quantification was based on the peak areas and calculated with the following equation % metabolite = metabolite peak area/total peak areas.

4.3.4 Identification of metabolites of abacavir analogues

Chemical structures of the compounds investigated are shown in Figure 4.3. The hepatic metabolism of each of these analogues was assessed. Each of the compounds (10μM) was incubated with human liver cytosol (1mg/ml) for 20 hours in the presence of the co-factor β-NAD (7.5mM). Where standards for the expected metabolites were not available a precursor ion scan targeted for the *m/z* 191 fragment was performed.

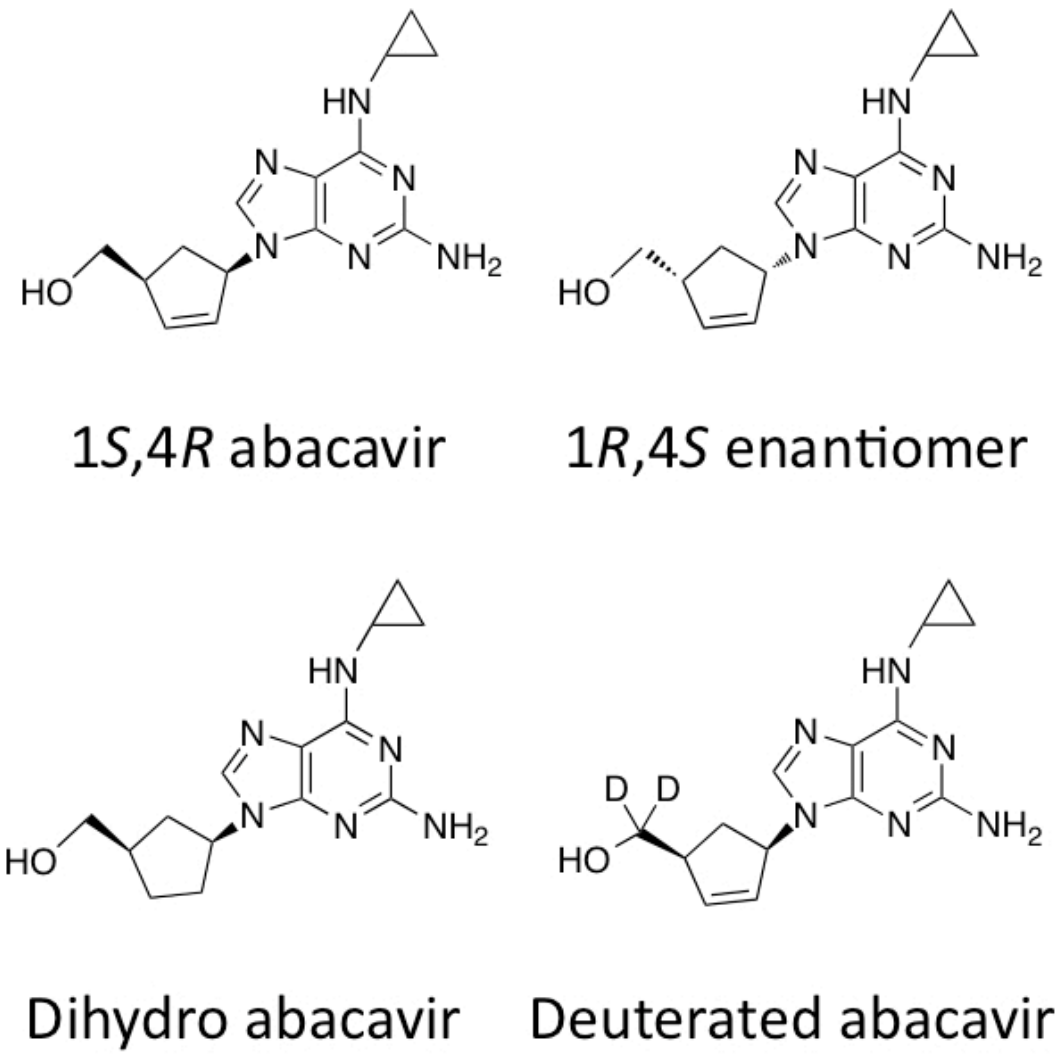

Figure 4.3 Abacavir analogues investigated in metabolism studies

4.3.5 Absolute quantification of abacavir turnover using deuterated abacavir as an internal standard.

The initial method was developed with 2-chloroadenosine as the internal standard, based on previous reports in the literature (Pruvost et al., 2009). During our investigations however the ionisation of 2-chloroadenosine proved to be inconsistent between runs. Following the synthesis of deuterated abacavir the potential of using it as a more robust internal standard for absolute quantification was investigated.

The chromatographic conditions and source parameters were as previously described (see 4.3.2). The following criteria were used to evaluate the performance of the method: linearity, recovery, matrix effects, sensitivity (LLOD and LLOQ), precision and accuracy.

4.3.5.1 Evaluation of linearity

Stock solutions (1mM) of abacavir and abacavir carboxylic acid were diluted to create a mixture of 8μM abacavir and 16μM abacavir carboxylic acid (Final volume 4ml) and serially diluted. All standards were made up in mobile phase (10mM ammonium acetate pH3.8 containing 5% acetonitrile). 1500μl of each standard was then added to 1500μl 400nM deuterated abacavir, which served as the internal standard. The final concentrations of the standard curve were therefore as shown in Table 4.2.

Table 4.2 Final concentrations of standards used in calibration curve

	Concentration (nM)		
Standard	Abacavir	Carboxylic acid	Deuterated abacavir
S1	4000	8000	200
S2	2000	4000	200
S3	1000	2000	200
S4	500	1000	200
S5	250	500	200
S6	100	200	200
S7	50	100	200
S8	25	50	200
S9	10	20	200
S10	5	10	200

The standard curve was generated by plotting the analyte:internal standard peak area ratio against the concentration of analyte. Six replicates of 10, 100 and 1000nM abacavir (100µl) were run in order to determine the accuracy and precision of the method and represented the 'neat' (100% response) standards when calculating recovery and matrix effects as described below. Standards were chosen to represent high, medium and low range concentrations.

The limits of detection and quantification (LLOD and LLOQ) were assigned based on a signal to noise ratio of 3:1 and 10:1 respectively.

4.3.5.2 Recoveries, precision and accuracy

To determine the recovery associated with the extraction method, human liver cytosol (1mg/ml; 100µl) was spiked with 10, 100 or 1000nM abacavir (100µl; n=6). Samples were then extracted by the standard protocol as described previously (see 4.3.2).

The recovery was calculated with the following equation:

Recovery (%) = (peak area of pre-spiked sample/peak area in neat standard) x 100

Accuracy was also determined by comparing the measured concentration (as determined by integration with the calibration curve) to the expected concentration:

Accuracy (%) = measured concentration/expected concentration x 100

The precision of the method was determined by calculating the coefficient of variation of the peak areas of the six replicate recovery samples:

Precision (%CV) = standard deviation/average x 100

4.3.5.3 Matrix effects

To determine whether there were any ion suppression or ion enhancement effects associated with the experimental matrix, extracted human liver cytosol was spiked with 100µl of 10, 100 or 1000nM abacavir solution (100µl; n=6).

Matrix effects were calculated with the following equation:

Matrix effect (%) = (peak area of post-spiked sample/peak area in neat standard) x100

4.3.5.4 Applications of validated LC-MS/MS method

The LC-MS/MS method was then applied to the comparative metabolism of abacavir and deuterated abacavir studies in human liver cytosol. Samples prepared as described previously using deuterated abacavir (Final concentration of IS = 200nM) or abacavir as the internal standard. Samples were diluted 1:10 prior to analysis to fit the measured concentration within the linear range of the calibration curve. The formation of carboxylic acid was expressed as a percentage and determined via the following equation:

% COOH = amount of COOH (nM)/ (amount of ABC (nM)+ amount of COOH) x 100

4.3.6 Assessment of the metabolising capacity of antigen-presenting cells

Sub-cellular fractions were generated from EBV-transformed B-cells. Approximately 1 x 10^9 EBV-transformed B-cells were pooled from 6 donors, pelleted, sonicated (4 x 15s) and centrifuged (9000g, 20min, 4˚C) to remove extracellular membranes. This was termed the S9 fraction. In order to generate a cytosol fraction, this extract was further centrifuged (48 000g, 1h, 4˚C) to remove the microsomes. Protein content was determined by the method of Bradford et al. (1976). A stock solution of bovine serum albumin was prepared (2mg/ml) in distilled water and diluted to generate a standard curve (Table 4.3). 10µl of each standard was added to a 96-well plate in duplicate. Samples were analysed at several different dilutions (e.g 1:5, 1:10, 1:50, 1:100, 1:250). Bradford reagent was diluted 1:5 in distilled water and added to wells (200µl).

Absorbance was then measured at 570nm. The standard curve was generated by firstly subtracting the absorbance in blank wells from all values. These blanked absorbances were then averaged across duplicates and plotted against the concentration of BSA. Sample dilutions that fell within the range of the standard curve were averaged to calculate the protein concentration.

Table 4.3 Dilution of bovine serum albumin standards for Bradford assay

Concentration (µg/ml)	Distilled water (µl)	Standard
2000	2000	2mg of BSA
1000	100	100µl of 2000µg/ml
500	100	100µl of 1000µg/ml
250	100	100µl of 500µg/ml
125	100	100µl of 250µg/ml
62.5	100	100µl of 125µg/ml
31.25	100	100µl of 62.5µg/ml

Incubations (5mg/ml; 500µl) were run in parallel and combined and concentrated prior to analysis. When required, the alcohol dehydrogenase inhibitor 4-methylpyrazole and the aldehyde dehydrogenase inhibitor disulfiram were used at 3mM and 0.5mM respectively. Supernatants from six incubations (total volume = 3ml; 15mg protein) were combined and evaporated to dryness before resuspending in 60µl mobile phase for LC-MS/MS analysis. The internal standard was added at a final concentration of 200nM. An aliquot of human liver cytosol (10mg/ml; 500µl) was also analysed in the experiment as a comparison. The percentage turnover in each incubation was calculated and corrected for the amount of protein.

4.3.7 Investigation of metabolising enzymes expressed by EBV-transformed B-cells.

Total RNA was extracted from EBV-transformed B-cells (5x 10^6) using the RNeasy mini kit as described previously (see 3.3.4.3). RNA was then reverse transcribed to cDNA using the RT^2-FirstStrand kit. RNA (1µg) was incubated with 2µl genomic DNA elimination reagents at 42˚C for 5 minutes (Final volume 10µl). A reverse transcription cocktail was prepared (containing random hexamers, oligo-dTs, reverse transcriptase and an external control mix) and 10µl was added to each 10µl aliquot of genomic DNA elimination mixture. This was incubated at 42˚C for 15 minutes. The reaction was terminated by increasing the temperature to 95˚C for 5 minutes. RNase free water (91µl) was then added to the resulting cDNA. The experimental cocktail was then prepared, comprising: 1350µl mastermix, 102µl cDNA, 1248µl H_2O. The mixture was briefly mixed and 25µl was added to each well of the PCR array plate. Thermal cycling was performed on an ABI 7000 thermal cycler for the following conditions: 95˚C for 10 minutes followed by 40 cycles of 95˚C for 15s and 60˚C for 1min. SYBR green was detected and recorded in each well. SYBR green is a fluorescent dye that binds to double stranded DNA. As the target is amplified the fluorescence increases accordingly. The amount of fluorescence during each cycle is quantified and displayed graphically. Relative quantification was achieved by determining the cycle at which the fluorescence reaches a threshold value (C_t).

4.3.8 Statistical analysis

Where biological replicates were available statistical significance was determined by one-way ANOVA.

4.4 Results

4.4.1 Chromatographic separation of abacavir standards

The chromatographic conditions described in 4.3.2 yielded good separation of abacavir (Rt = 14.98 min), abacavir carboxylic acid (Rt = 13.73 min) and the internal standard 2-chloroadenosine (Rt =10.15 min) (Figure 4.4). This method was applied for the following investigations.

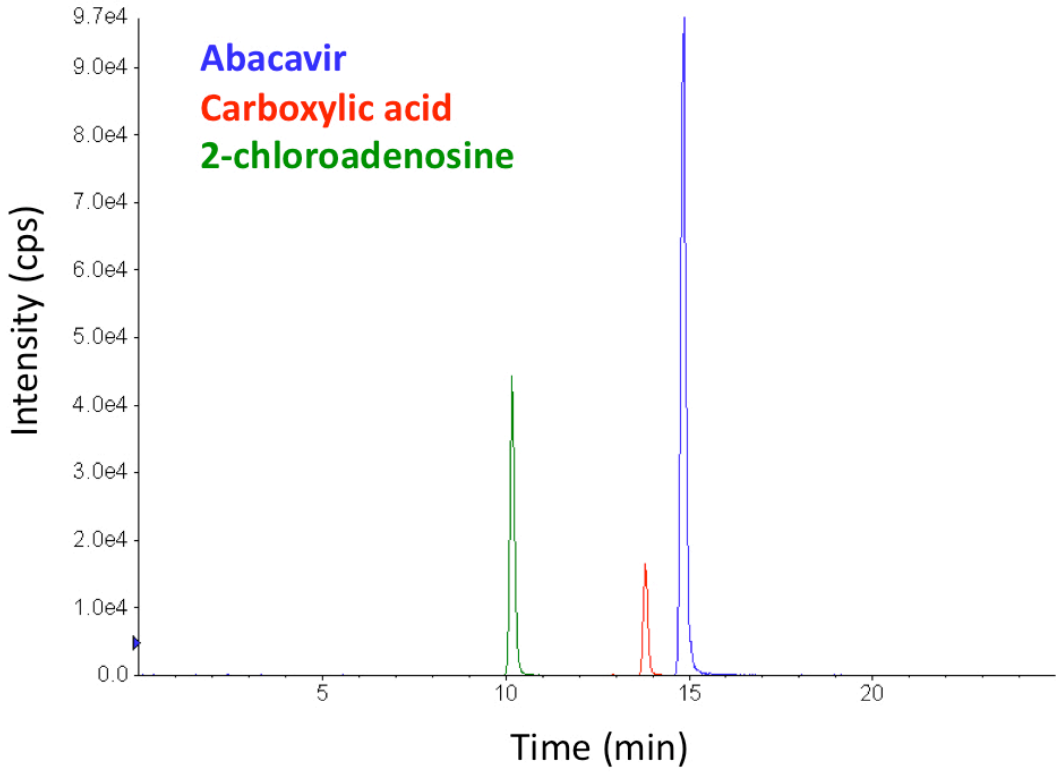

Figure 4.4 Chromoatographic separation of standards. Solution contained 2µM abacavir, 4µM carboxylic acid and 2µM 2-chloroadenosine.

4.4.2 Abacavir metabolism in human liver cytosol

The LC-MS/MS conditions described were used to investigate the metabolism of abacavir in human liver cytosol. Abacavir was metabolised to three isomeric carboxylic acids following incubation with human liver cytosol (Figure 4.5A). The chromatographic conditions resulted in good separation of abacavir and the carboxylic acids. It was a relatively slow reaction however, requiring up to 20 hour incubation in order to yield approximately 50% product (Figure 4.5B). Abacavir metabolism was inhibited by the addition of the alcohol dehydrogenase inhibitor 4-methylpyrazole (Figure 4.6). At the maximum concentration of 4-methylpyrazole (1000μM) 91.7% (±3.9) inhibition of alcohol dehydrogenase was observed ($p<0.001$ vs.. control; one-way ANOVA). Trapping of the intermediary aldehyde as an oxime derivative was observed following incubation with methoxylamine (Figure 4.7A and B; R_t= 22.99min, MH^+=314 and Figure 4.8).

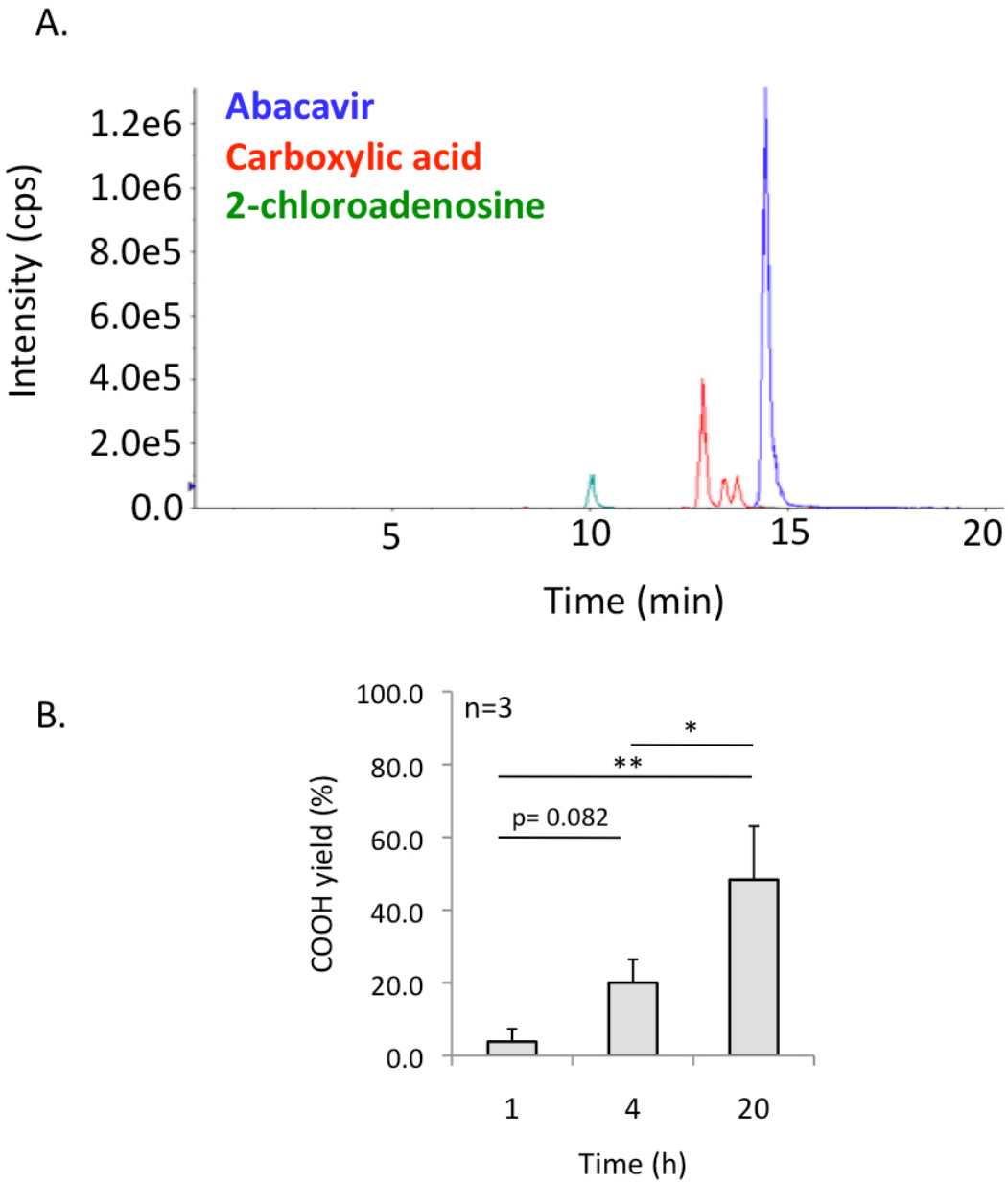

Figure 4.5 Abacavir is metabolised to three isomeric carboxylic acids in human liver cytosol A) Representative chromatogram following 20h incubation of abacavir with human liver cytosol B) Relative formation of carboxylic acid Data represent mean and standard deviation of three independent experiments (* = $p<0.05$, ** = $p<0.001$; One way ANOVA).

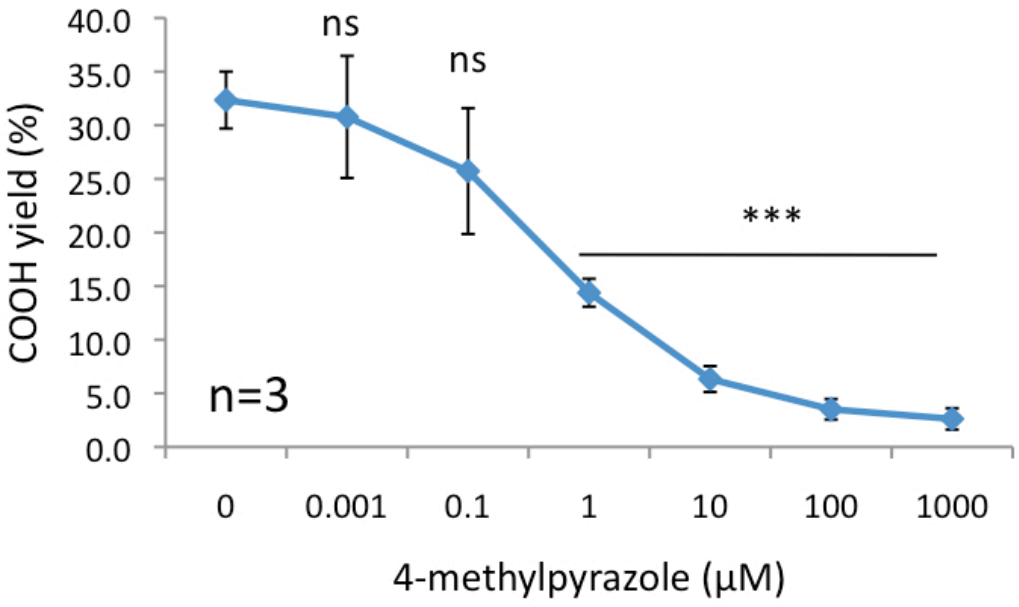

Figure 4.6 Inhibition of abacavir metabolism by 4-methylpyrazole. Abacavir was incubated with human liver cytosol in the presence of the indicated concentration 4-methylpyrazole for 20h. Data represent mean and standard deviation of 3 independent experiments (ns= not significant, *** = $p<0.001$; control vs. treated; one-way ANOVA)

4.4.3 Identification of metabolites formed from abacavir analogues in human liver cytosol

Both enantiomers of abacavir were similarly metabolised to isomeric carboxylic acids (Figure 4.9 A and C) and this was blocked by 4-methylpyrazole (Figure 4.9 B and D). As predicted from the mechanisms of aldehyde isomerisation proposed by Walsh et al. (2002) namely epimerisation and double bond migration, dihydro abacavir was metabolized to a single carboxylic acid in human liver cytosol (Figure 4.10). Dihydro abacavir (m/z 289) eluted at 15.2 minutes. A more polar product, which eluted at 14.8 minutes, had a m/z of 303 Da consistent with the formation of a carboxylic acid (Figure 4.10). Metabolism was blocked by the addition of 4-methylpyrazole (data not shown).

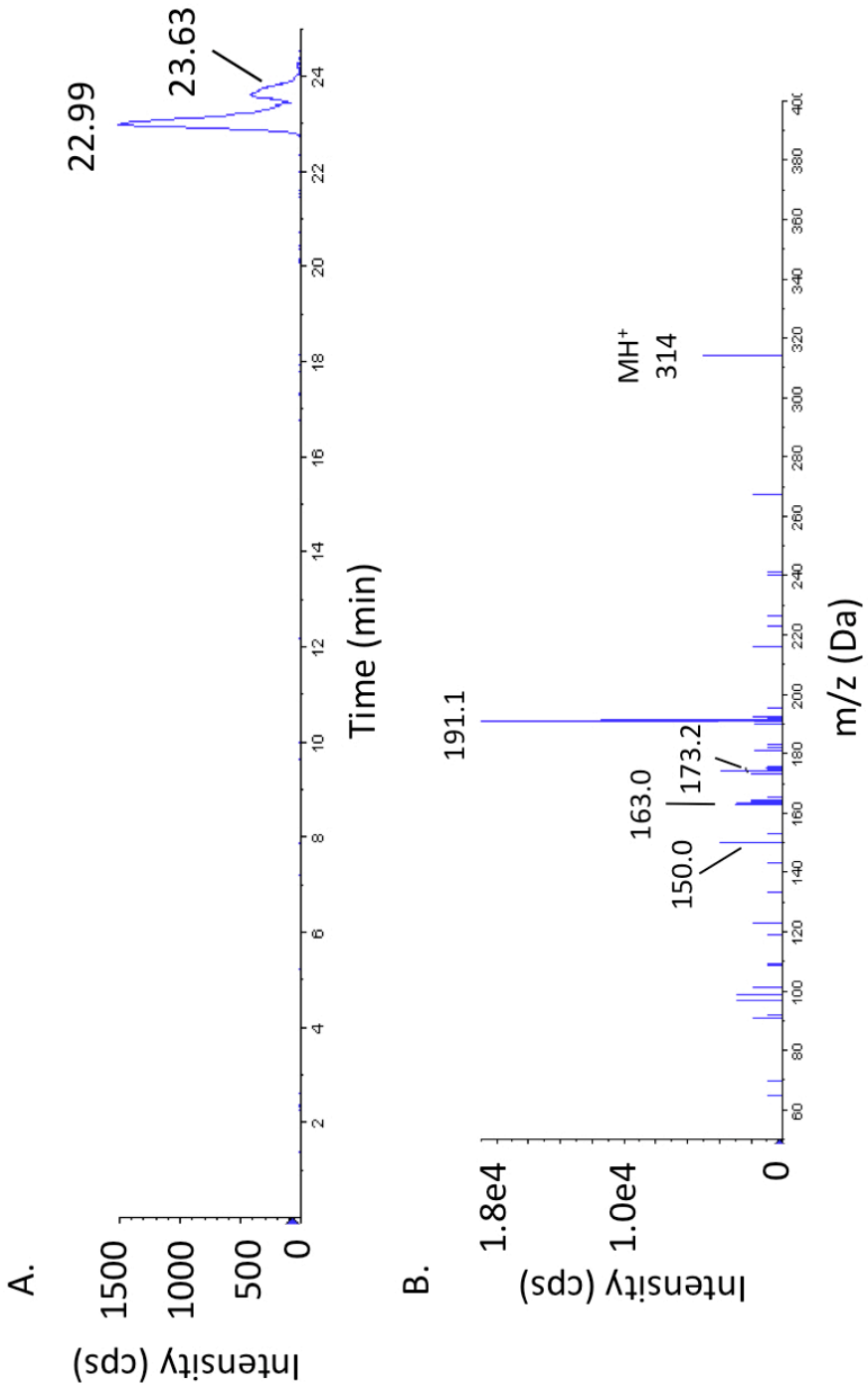

Figure 4.7 Reaction of abacavir aldehyde with methoxylamine. Abacavir was incubated with human liver cytosol in the presence of 10mM methoxylamine. A) Chromatogram of product formed (R_t = 22.99 minutes) B) Mass spectrum of product. Molecular ion m/z = 314Da

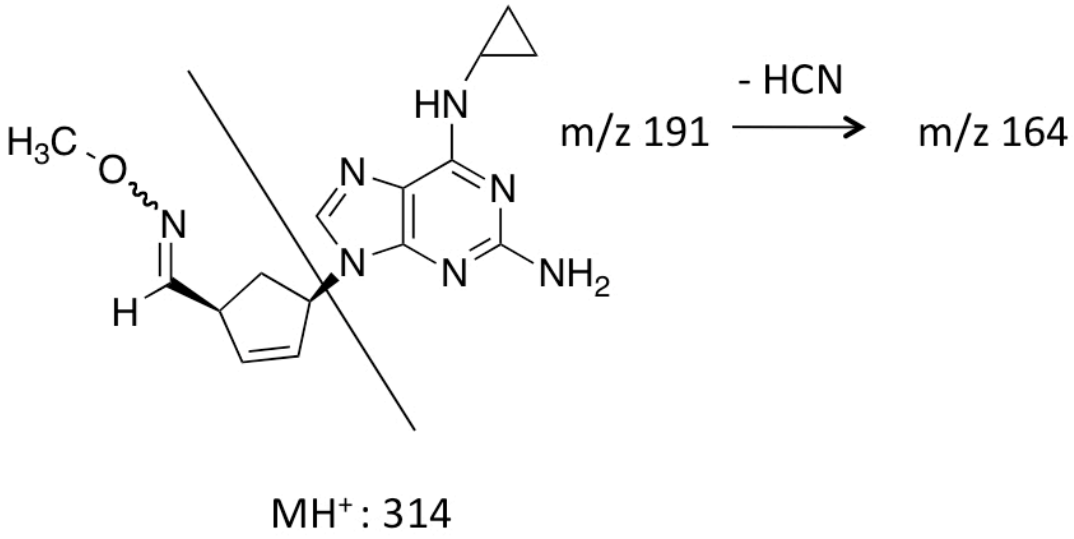

Figure 4.8 Incubation of abacavir with human liver cytosol in the presence of 1mM methoxylamine yields an oxime product MH^+ =314. Ions with a m/z of 191 and 164 Da were also observed (see Figure 4.7). Adapted from (Walsh et al., 2002).

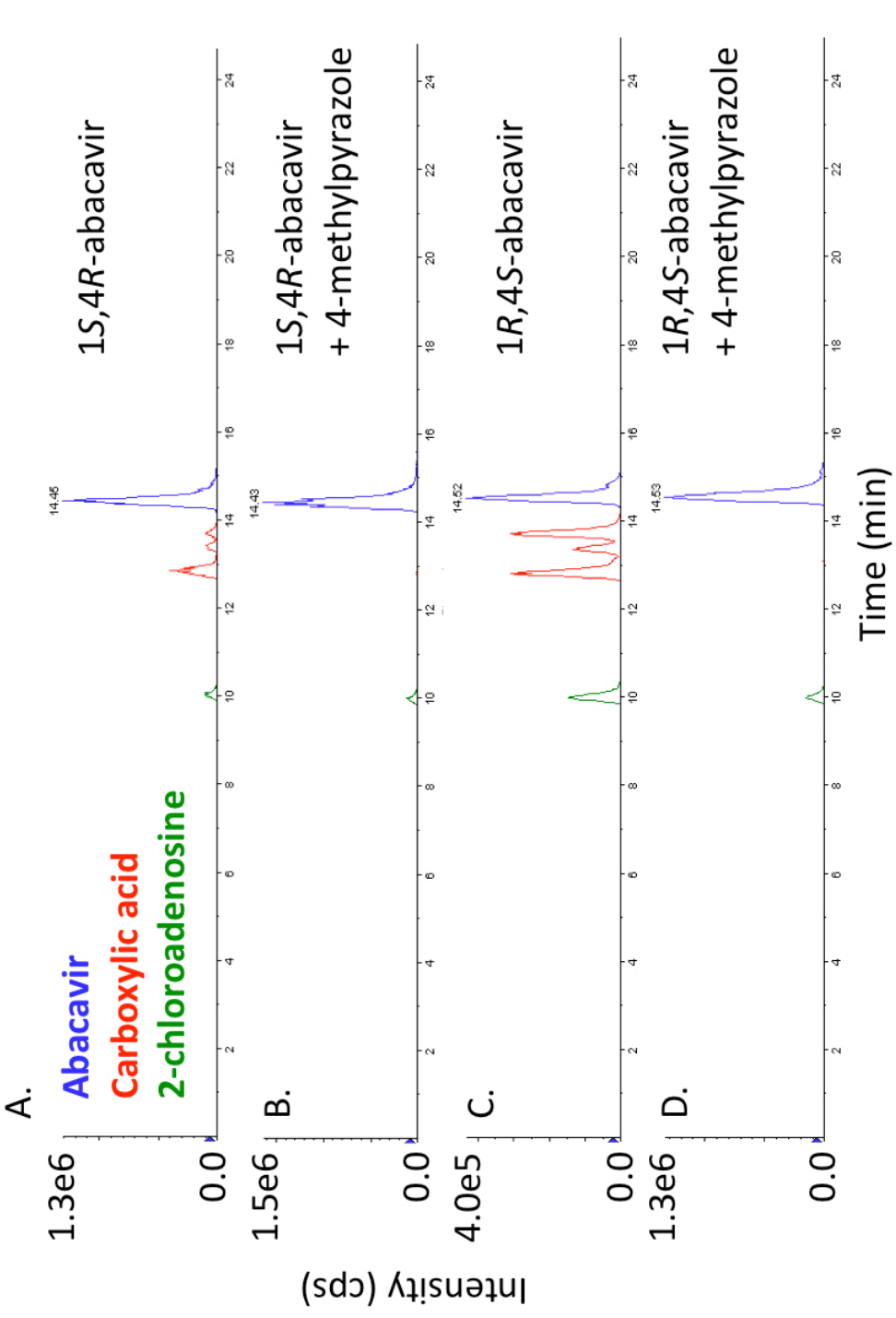

Figure 4.9 Comparative metabolism of abacavir enantiomers in human liver cytosol. Each of the compounds was incubated with human liver cytosol for 20h in the presence (B,D) or absence (A,C) of 600µM 4-methylpyrazole.

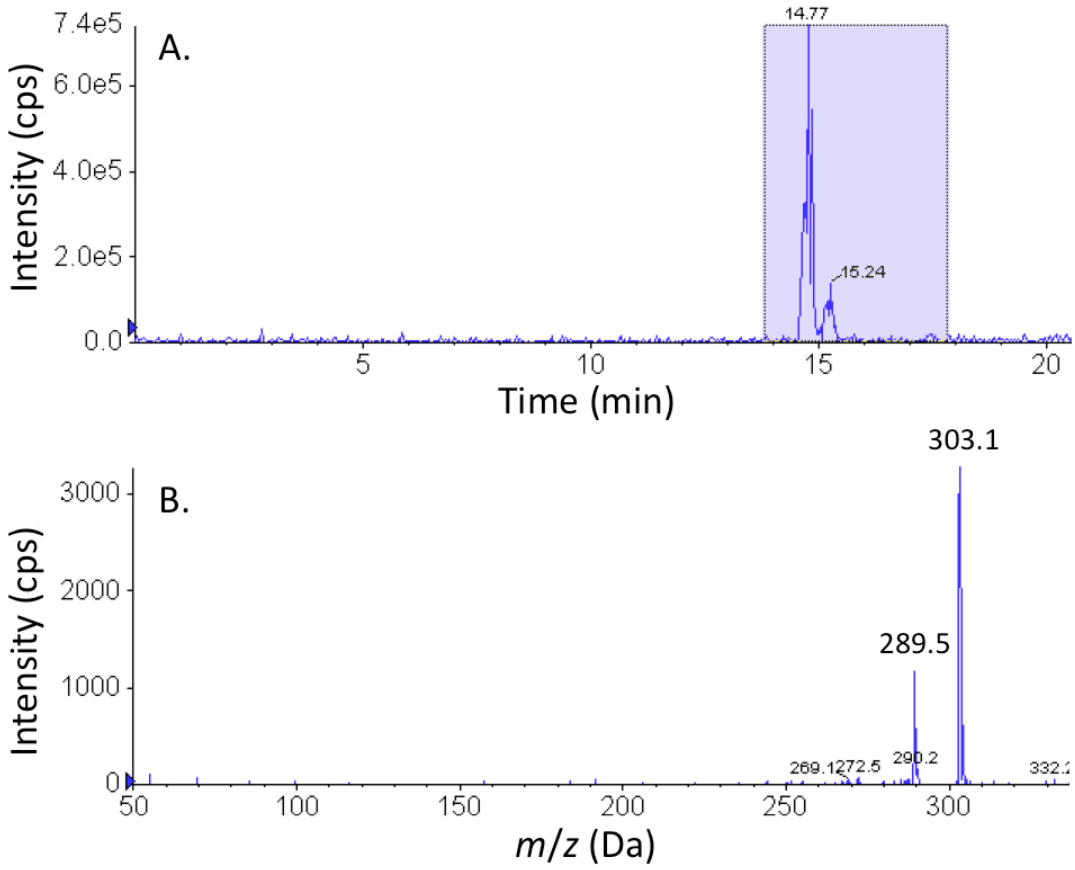

Figure 4.10 Metabolism of dihydro abacavir in human liver cytosol. As no standards of dihydro metabolites were available, a precursor ion scan was performed for species containing a fragment with a m/z of 191 (see Figure 4.8). A) Chromatogram Rt 14.8 minutes= carboxylic acid; 15.2 minutes = dihydro abacavir B) Precursor ions m/z 289.5 = dihydro abacavir; 303.1 = corresponding carboxylic acid.

4.4.4 Absolute quantification of abacavir turnover using deuterated abacavir as an internal standard.

At the time of the establishment of the LC-MS/MS method 2-chloroadenosine was selected as an internal standard as it had been previously reported in the literature (Pruvost et al., 2009). During the development of the methodology, 2-chloroadenosine proved to be an unsuitable internal standard to be used for absolute quantification due to its poor ionisation properties. As the deuterated

version of abacavir became available it was decided to utilise this compound as a more appropriate internal standard for the validation of the quantitative method. Isotope labelled internal standards are also thought to reduce the influence of any matrix effects (Atzrodt et al., 2007). Figure 4.11 shows the chromatogram of abacavir, abacavir carboxylic acid and deuterated abacavir under the standard LC conditions.

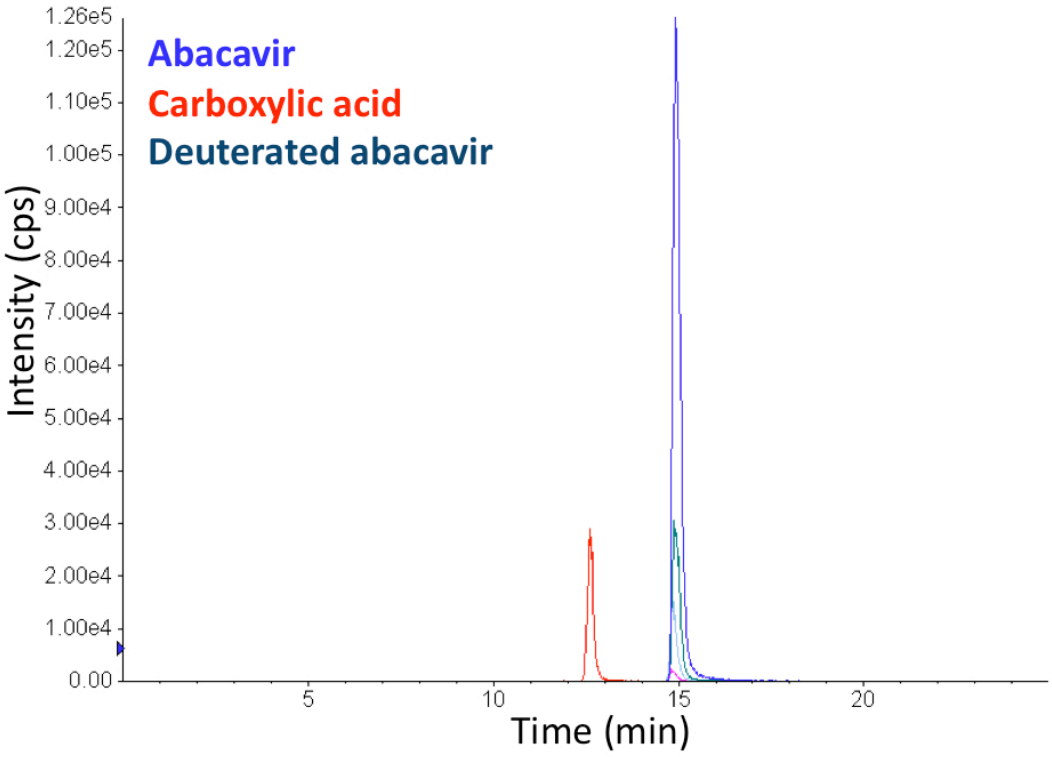

Figure 4.11 Chromatographic separation of abacavir (1µM), carboxylic acid (2µM) and 200nM deuterated abacavir internal standard.

4.4.4.1 Evaluation of linearity

Standard curves for both abacavir and carboxylic acid were generated as described previously in 4.3.5.1. The analyte to internal standard ratio was plotted against the concentration of analyte. Standard curves were linear over the ranges examined (abacavir =5-2000nM, carboxylic acid =10-4000nM,)(abacavir R^2 = 0.997 ± 0.003 slope = 0.005 ± 0.002; carboxylic acid R^2 = 0.999 ± 0.000 slope = 0.001 ± 0.000). Typical calibration curves are shown in Figure 4.12 A and B.

In order to provide a more accurate quantification of the lowest concentrations (Abacavir <250nM; Carboxylic acid <500nM) separate 'low-range' calibration curves were also generated. The LLOD and LLOQ were 0.075nM and 0.3nM for abacavir and 2.5nM and 10nM for the carboxylic acid.

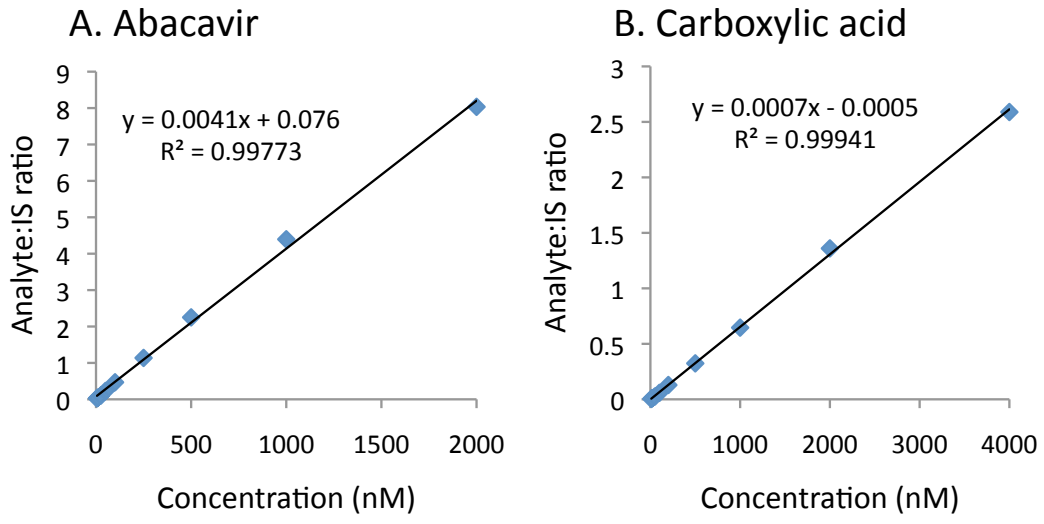

Figure 4.12 Abacavir (A) and carboxylic acid (B) standard curves utilised for absolute quantification. Deuterated abacavir was used as the internal standard (200nM).

4.4.4.2 Intra/inter day precision and accuracy

The reproducibility of the method was assessed using a number of parameters. Three separate experiments were performed on separate days and six replicates of each concentration were analysed each time.

Recovery gives an indication of the extraction efficiency. Recoveries on different days are shown in Table 4.4. Precision gives a measure of the repeatability of the measurements and accuracy how close the measured value is to the known concentration. Intra and inter day precision for abacavir and carboxylic acid are also shown in table 4.4. The accuracy of calculated concentrations ranged from 86.6 to 112% for abacavir and from 86.4 to 113.3% for the carboxylic acid.

Matrix effects relate to any components within the experimental matrix, that may co-elute/interfere with the response of the analytes of interest leading to enhancement (values >100%) or suppression (values <100%) of the analyte signal. For abacavir, matrix effects ranged between 92.7 and 110.1% (Table 4.5). For the carboxylic acid, matrix effects of between 91.8 and 112.8% were observed (Table 4.5).

Table 4.4 Intra and inter assay accuracy and precision

Intra-assay variability (1) n=6

Analyte	Nominal concentration (nM)	Recovery (%)	Precision CV (%)	Accuracy (%)
ABC	1000	92.3	6	112
	100	105.7	10	101.6
	10	118.5	15.5	105.6
COOH	2000	91.3	6.7	89
	200	104.2	8.9	113
	20	122.8	12.7	100

Intra-assay variability (2) n=6

Analyte	Nominal concentration (nM)	Recovery (%)	Precision CV (%)	Accuracy (%)
ABC	1000	102	2	107.2
	100	104.6	4.6	99.6
	10	89.6	2.4	109.8
COOH	2000	105.5	2	113.1
	200	108.7	5.4	108.6
	20	94.4	9.5	108.3

Intra-assay variability (3) n=6

Analyte	Nominal concentration (nM)	Recovery (%)	Precision CV (%)	Accuracy (%)
ABC	1000	93.7	1.2	105.6
	100	86.9	2	101
	10	96.5	4	86.6
COOH	2000	103.1	1.5	102.9
	200	89.1	3.9	91.1
	20	101.2	9.5	86.4

Inter-assay variability n=3

Analyte	Nominal concentration (nM)	Recovery (%)	Precision CV (%)	Accuracy (%)
ABC	1000	95.8	5.5	109
	100	99.1	10.7	101
	10	101.5	14.9	101
COOH	2000	96.8	7.6	102
	200	100.7	10.2	104
	20	106.1	14.0	98

Table 4.5 Matrix effects associated with human liver cytosol

		Matrix effect (%)			
Analyte	Nominal concentration (nM)	1	2	3	Overall
ABC	1000	100.7	102.1	98.5	100.4
	100	92.7	109.7	98.9	100.4
	10	110.1	103.3	102.2	105.2
COOH	2000	100.2	106.5	103.8	103.5
	200	91.8	111.9	99.3	101.0
	20	112.8	102.4	106.6	107.3

4.4.4.3 Absolute quantification of abacavir and abacavir carboxylic acid in human liver cytosol

The quantitative method described above was applied to determine the concentrations of carboxylic acid and abacavir in human liver cytosol. Figure 4.13 (black line) shows the formation of carboxylic acid over 24 hours. Deuterated abacavir was also metabolised to three isomeric carboxylic acids. Given that introduction of deuterium can slow the rate of metabolism (Mutlib, 2008), a small time-course was investigated (Figure 4.13, grey line). A reduction in the rate of the reaction was observed ($p<0.001$; abacavir vs. deuterated).

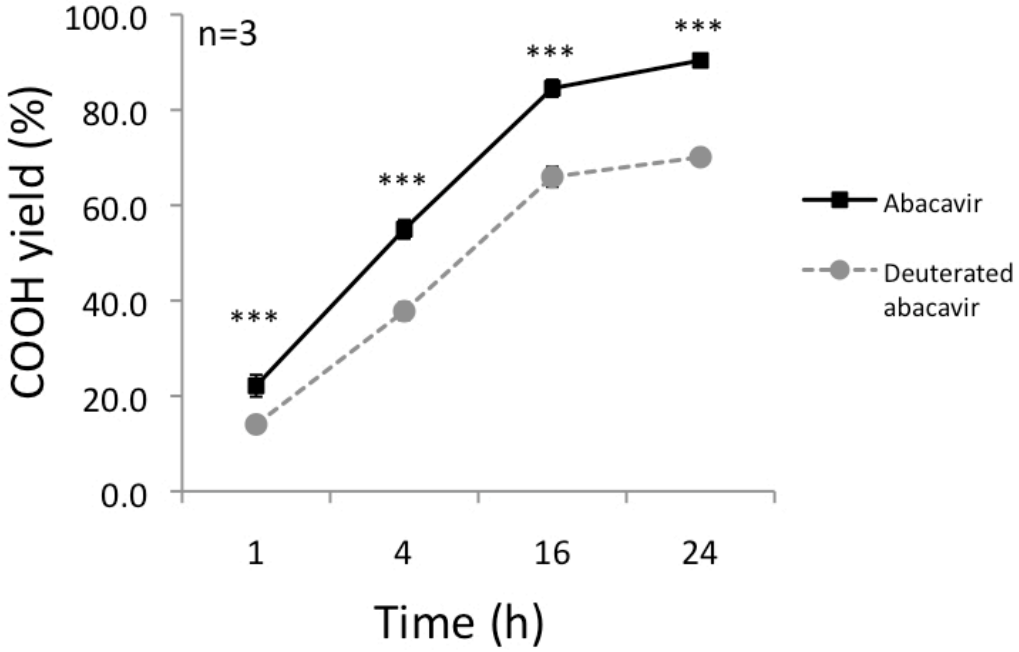

Figure 4.13 Comparative metabolism of abacavir and deuterated abacavir in human liver cytosol. Quantification was based on absolute quantification. Human liver cytosol (1mg/ml) was incubated with each of the compounds (10μM) in the presence of the co-factor NAD for the indicated time-points. Data represent the mean ± standard deviation of three experiments (*** = $p<0.001$; one-way ANOVA)

4.4.4.4 Investigation of abacavir metabolism in human EBV-transformed B-cells

Cytosol and S9 fractions were generated from human EBV-transformed B-cells. Following 20h incubation with 10μM abacavir in the presence of NAD oxidation to three isomeric carboxylic acids was observed (Figure 4.14). The amount of carboxylic acid was quantified using a standard curve (ranging from 10-4000nM) and expressed as a percentage of the total amount of abacavir and metabolites (Table 4.6). Preliminary attempts were made to block the metabolism of abacavir with 4-methylpyrazole however carboxylic acid metabolites were still formed (Figure 4.15). The aldehyde dehydrogenase inhibitor disulfiram inhibited the metabolism of abacavir by approximately 70%.

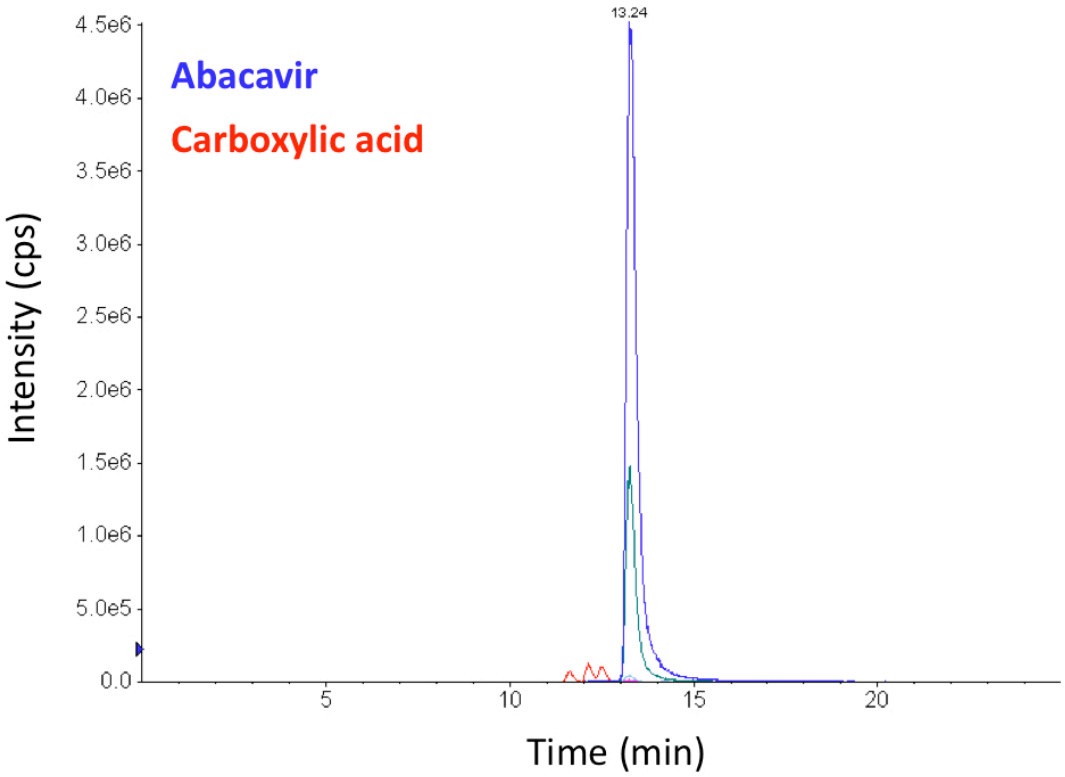

Figure 4.14 Formation of carboxylic acids observed in cytosolic fraction isolated from human EBV-transformed B-cells.

4.4.5 Investigation of metabolising enzymes expressed by EBV-transformed B-cells

RNA isolated from EBV-transformed B-cells was subjected to PCR array analysis. The genes investigated are listed in Table 4.7, alongside the average C_t values obtained from two replicate samples. The C_t value is an indication of the PCR cycle at which the fluorescent signal reaches a threshold intensity, hence the lower the number the more abundant the target. Where no amplification of the target was observed within the 40 cycles an 'undetermined' call was assigned.

Table 4.6 Comparative metabolism of abacavir in human liver cytosol and immune cell preparations following 20h incubation. The concentration of carboxylic acid was determined by using the equation from linear regression of the standard curve and converted to percentage yield. Values were corrected for the amount of protein in the incubation.

	Formation of carboxylic acid (%)	Amount of protein (mg)	Relative metabolic capacity (%)
Liver	67	5	67
EBV-transformed B -cells			
Cytosol	11	15	4
S9	6	15	2

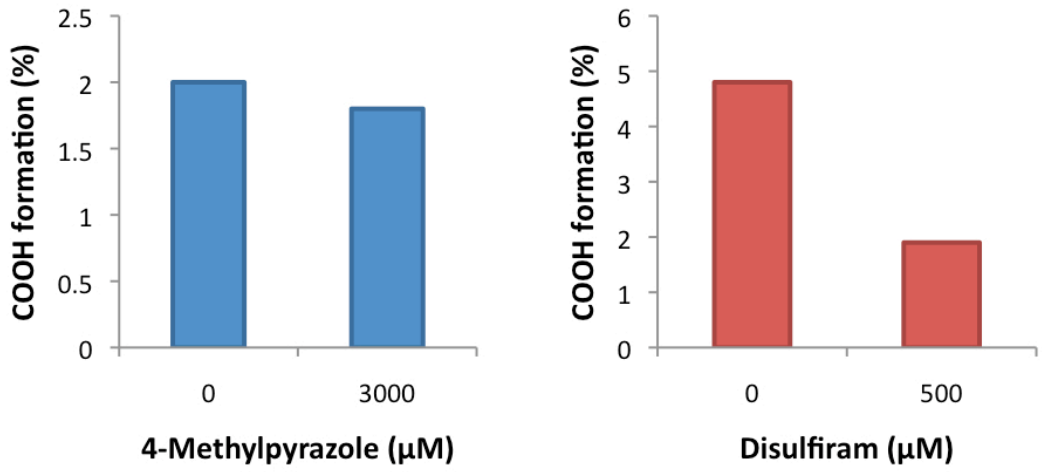

Figure 4.15 Preliminary attempts to block metabolism in S9 fraction isolated from human EBV-transformed B-cells with 4-methylpyrazole and disulfiram.

Table 4.7 Expression of drug metabolising enzymes in EBV-transformed B-cells.

RNA transcript	Average Ct
Arylacetamide deacetylase (esterase)	Undetermined
Alcohol dehydrogenase 1A (class I), alpha polypeptide	Undetermined
Alcohol dehydrogenase 1B (class I), beta polypeptide	35.2
Alcohol dehydrogenase 1C (class I), gamma polypeptide	36.5
Alcohol dehydrogenase 4 (class II), pi polypeptide	37.9
Alcohol dehydrogenase 5 (class III), chi polypeptide	29.0
Alcohol dehydrogenase 6 (class V)	37.9
Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	35.0
Aldehyde dehydrogenase 1 family, member A1	35.7
Aldehyde dehydrogenase 1 family, member A2	36.3
Aldehyde dehydrogenase 1 family, member A3	34.2
Aldehyde dehydrogenase 1 family, member B1	38.1
Aldehyde dehydrogenase 2 family (mitochondrial)	33.8
Aldehyde dehydrogenase 3 family, member A1	Undetermined
Aldehyde dehydrogenase 3 family, member A2	29.3
Aldehyde dehydrogenase 3 family, member B1	36.6
Aldehyde dehydrogenase 3 family, member B2	37.0
Aldehyde dehydrogenase 4 family, member A1	32.5
Aldehyde dehydrogenase 5 family, member A1	31.8
Aldehyde dehydrogenase 6 family, member A1	29.2
Aldehyde dehydrogenase 7 family, member A1	32.6
Aldehyde dehydrogenase 8 family, member A1	Undetermined
Aldehyde dehydrogenase 9 family, member A1	26.7
Carboxyl ester lipase (bile salt-stimulated lipase)	33.3
Cytochrome P450, family 11, subfamily A, polypeptide 1	38.4
Cytochrome P450, family 11, subfamily B, polypeptide 1	36.2
Cytochrome P450, family 11, subfamily B, polypeptide 2	38.6
Cytochrome P450, family 17, subfamily A, polypeptide 1	Undetermined
Cytochrome P450, family 19, subfamily A, polypeptide 1	34.2
Cytochrome P450, family 1, subfamily A, polypeptide 1	33.8
Cytochrome P450, family 1, subfamily A, polypeptide 2	Undetermined
Cytochrome P450, family 1, subfamily B, polypeptide 1	26.2
Cytochrome P450, family 21, subfamily A, polypeptide 2	39.1
Cytochrome P450, family 24, subfamily A, polypeptide 1	Undetermined
Cytochrome P450, family 26, subfamily A, polypeptide 1	33.5
Cytochrome P450, family 26, subfamily B, polypeptide 1	38.8
Cytochrome P450, family 26, subfamily C, polypeptide 1	39.8
Cytochrome P450, family 27, subfamily A, polypeptide 1	39.8
Cytochrome P450, family 27, subfamily B, polypeptide 1	34.0
Cytochrome P450, family 2, subfamily A, polypeptide 13	35.4
Cytochrome P450, family 2, subfamily B, polypeptide 6	35.6
Cytochrome P450, family 2, subfamily C, polypeptide 18	35.4
Cytochrome P450, family 2, subfamily C, polypeptide 19	33.1
Cytochrome P450, family 2, subfamily C, polypeptide 8	35.1
Cytochrome P450, family 2, subfamily C, polypeptide 9	Undetermined
Cytochrome P450, family 2, subfamily D, polypeptide 6	32.5

Table 4.7 continued.

RNA transcript	Average Ct
Cytochrome P450, family 2, subfamily E, polypeptide 1	Undetermined
Cytochrome P450, family 2, subfamily F, polypeptide 1	39.7
Cytochrome P450, family 2, subfamily R, polypeptide 1	30.1
Cytochrome P450, family 2, subfamily S, polypeptide 1	35.4
Cytochrome P450, family 2, subfamily W, polypeptide 1	36.0
Cytochrome P450, family 3, subfamily A, polypeptide 4	Undetermined
Cytochrome P450, family 3, subfamily A, polypeptide 43	Undetermined
Cytochrome P450, family 3, subfamily A, polypeptide 5	34.7
Cytochrome P450, family 3, subfamily A, polypeptide 7	38.0
Cytochrome P450, family 4, subfamily A, polypeptide 11	36.9
Cytochrome P450, family 4, subfamily A, polypeptide 22	Undetermined
Cytochrome P450, family 4, subfamily B, polypeptide 1	37.5
Cytochrome P450, family 4, subfamily F, polypeptide 11	36.0
Cytochrome P450, family 4, subfamily F, polypeptide 12	36.3
Cytochrome P450, family 4, subfamily F, polypeptide 2	35.7
Cytochrome P450, family 4, subfamily F, polypeptide 3	37.7
Cytochrome P450, family 4, subfamily F, polypeptide 8	38.2
Cytochrome P450, family 7, subfamily A, polypeptide 1	35.1
Cytochrome P450, family 7, subfamily B, polypeptide 1	29.5
Cytochrome P450, family 8, subfamily B, polypeptide 1	Undetermined
Dehydrogenase/reductase (SDR family) member 2	39.8
Dihydropyrimidine dehydrogenase	28.5
Esterase D	24.4
Flavin containing monooxygenase 1	39.8
Flavin containing monooxygenase 2 (non-functional)	37.7
Flavin containing monooxygenase 3	38.7
Flavin containing monooxygenase 4	32.8
Flavin containing monooxygenase 5	34.0
Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	33.4
Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	31.9
Hydroxysteroid (17-beta) dehydrogenase 10	28.7
Monoamine oxidase A	36.3
Monoamine oxidase B	Undetermined
Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	33.2
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	33.7
Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	28.9
Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	28.1
Xanthine dehydrogenase	37.6
Beta-2-microglobulin	23.1
Hypoxanthine phosphoribosyltransferase 1	25.2
Ribosomal protein L13a	23.9
Glyceraldehyde-3-phosphate dehydrogenase	21.7
Actin, beta	19.8

4.5 Discussion

The formation of reactive metabolites is often associated with drug toxicity. Abacavir administration is associated with hypersensitivity reactions in up to 8% of patients undergoing therapy. Previous investigations have suggested the formation of a reactive aldehyde metabolite, which has the potential to bind to proteins (Walsh et al., 2002, Charneira et al., 2011) and could lead to immunogenicity through a classical hapten mechanism. In order to determine whether T-cells in *in vitro* assays are exposed to this metabolite an LC-MS/MS method was developed to quantify abacavir and its metabolites.

The LC-MS/MS conditions investigated yielded good chromatographic separation of abacavir and its carboxylic acid metabolite. Firstly the method was applied to human liver cytosol given that the metabolism of abacavir has previously been characterised in this matrix and it provides a rich source of alcohol dehydrogenase. Abacavir was metabolised to three isomeric carboxylic acids via an aldehyde intermediate that could be trapped with methoxylamine. Formation of the carboxylic acids could therefore be used to indicate that the aldehyde had been produced.

A number of analogues of abacavir were synthesised in order to investigate metabolism and the specificity of drug-responsive T-cell clones. Modifications to the structure of abacavir may impact upon the stereochemistry of the molecule and hence the direct interaction between abacavir and the MHC/TCR or may alter the metabolism of the compound. The stereochemistry of abacavir is known to be important for its pharmacological activity (Faletto et al., 1997) and

so the stereoselectivity of alcohol dehydrogenase was also investigated. Pharmaceutical abacavir is a single enantiomer in the 1*S*,4*R* configuration. This is because the alternative 1*R*,4*S* configuration lacks pharmacological activity due to enantioselectivity at the active site of the enzyme adenosine phosphotransferase (Faletto et al., 1997). Analysis of the metabolism of pharmaceutical 1*S*,4*R* abacavir and the alternative 1*R*,4*S* enantiomeric form to the corresponding carboxylic acids by human liver cytosol confirmed that both molecules are substrates for alcohol dehydrogenase (Walsh et al., 2002). This would indicate that any differences in T-cell response would be due to the altered stereochemistry of the parent drug/metabolites and not due to impaired metabolism (see Chapter 5). Carbovir, the molecule on which abacavir is based is also metabolised by alcohol dehydrogenase. Both enantiomers are metabolised in rat liver cytosol but the (+)-enantiomer (1S,4R) is favoured by 6-7 fold (Patanella and Walsh, 1992). Our investigation however was merely qualitative.

Metabolism experiments showed that dihydro abacavir formed a single carboxylic metabolite when incubated with human liver cytosol. Double bond migration is involved in the isomerisation of abacavir metabolites hence the removal of the double bond results in the formation of a single isomer of dihydro carboxylic acid. Previously dihydro abacavir has been reported to be more susceptible to metabolism but produce fewer non-extractable residues than abacavir (Walsh et al., 2002). It was this observation that implicated the double bond in the mechanism of protein conjugation.

In addition, deuterated abacavir was synthesised as a potential tool for examining the role of metabolism in the abacavir-specific T-cell response. The cleavage of carbon-deuterium bonds requires higher activation energy than carbon-hydrogen bonds. In preliminary investigations, deuterated abacavir was metabolised to three isomeric carboxylic acids but at a slower rate than abacavir. This is termed a kinetic isotope effect. The full characterisation of this effect requires further studies that are outside the scope of this thesis. In particular, calculation of the reaction rate constants.

The availability of deuterated abacavir also presented an opportunity to use it as an internal standard in the absolute quantification of abacavir and metabolites from *in vitro* incubations. Isotope-labelled internal standards generally have the same retention time and ionisation characteristics as the analyte of interest but can be identified by their increased mass. If the signal overlap is kept to a minimum a quantitative method can be established (Atzrodt et al., 2007). Isotope labelled internal standards are also thought to reduce the influence of any matrix effects (Atzrodt et al., 2007). This is because they elute at the same time as the analyte of interest meaning that both the internal standard and analyte are affected proportionally.

Firstly quality control samples were generated in order to establish that the method could be reliably used to quantify abacavir and the carboxylic acid in samples. The calibration curves were linear (R^2>0.99) over the ranges examined for abacavir (5-2000nM) and carboxylic acid (10-4000nM). The LLOD and LLOQ were 0.075nM and 0.3nM for abacavir and 2.5nM and 10nM for the carboxylic

acid indicating that the method had the required sensitivity for the samples to be analysed. Intra and inter day precision for abacavir ranged from 2 to 15% and 5.5 to 14.9 % respectively. Accuracy was found to be between 86.6 and 112%. For abacavir carboxylic acid intra and inter day precision ranged from 1.5 to 12.7% and 7.6 to 14%. Accuracy was between 86.4 and 113.3%. Recoveries ranged between 86.9-118.5% for abacavir and 89.1-122.8% for carboxylic acid.

Matrix effects generally refer to ion suppression or ion enhancement, which can interfere with the accurate quantitation of the analyte of interest. Matrix effects can be a result of co-eluting contaminants from the biological matrix (e.g salts, triglycerides, amines), though this is not always the case. Indeed all stages of the sample preparation can influence the ionisation of the sample, even down to the plasticware used (Mei et al., 2003). They can be particularly problematic when complex biological matrices are investigated. In the method developed here no significant matrix effects were observed. For abacavir matrix effects ranged between 93-110% and this was similar to those calculated for the carboxylic acid of between 92 and 113%. Matrix effects were particularly evident at the lowest concentrations of both analytes though this may be due to the lower sensitivity of the method at this level.

These fulfilled the criteria provided by the FDA (US Department of Health and Human Services, 2001) for the validation of bioanalytical methods. Hence, the validated LC-MS/MS method was considered reproducible and reliable quantification for the studies described in this thesis.

Once validated, the quantitative method was firstly applied to human liver cytosol incubations. The metabolic activity of immune cells was then investigated. Carboxylic acid metabolites were detected following 20h incubation of abacavir with an S9 fraction generated from EBV-transformed B-cells. This was however dependent on the concentration of samples prior to analysis. The protein content was also increased for each incubation due to the suspected low expression of metabolising enzymes. Oxidative metabolism was also detected in a cytosolic fraction prepared from EBV-transformed B-cells.

A small amount of carboxylic acid is formed, however it is unclear what proportion of the aldehyde this represents and how much is subsequently available to bind to proteins. Recent *in vivo* studies have characterised abacavir binding to haemoglobin however it is unclear whether these adducts are capable of stimulating T-cells (Charneira et al., 2012). It is likely that these *in vivo* adducts are formed as a result of hepatic metabolism. One of the main arguments for a metabolism-independent mechanism of T-cell activation has been that B and T-cells do not express class I alcohol dehydrogenase (Adam et al., 2012), however given that oxidative metabolism does indeed occur in these cells this may not be important. In fact, the preliminary observation that metabolism is not blocked by 4-methylpyrazole might indicate an as yet undefined alcohol dehydrogenase-independent route of metabolism. Further studies are required in order to confirm this.

The alcohol dehydrogenase inhibitor 4-methylpyrazole inhibits class I and II ADH efficiently. Class III ADH which is widely expressed in many tissues is

however insensitive to 4-methylpyrazole. It has a rather different substrate specificity to the class I and II ADHs and metabolises ethanol very poorly (Wagner et al., 1984). Pyrazole had very little effect on metabolism of ethanol in cytosol isolated from rat skin (Lockley et al., 2005). Similarly cytosol isolated from human skin retained 38% of control activity following pyrazole treatment (Cheung et al., 2003). ADH class III has not previously been shown to metabolise abacavir however (Walsh et al., 2002).

Preliminary gene expression analysis indicates that the RNA of a number of drug metabolising enzymes is expressed in EBV-transformed B-cells. ADH5 was expressed at a high level as reported previously (Adam et al., 2012). Enzymes expressed at a similar level include a number of aldehyde dehydrogenases, CYPs 1B1, 2C19 and 7B1, hydroxysteroid 17-β dehydrogenase, esterase D and ubiquitin thiolesterases. It is unclear whether any of these have the potential to metabolise abacavir. It would appear however that a cytosolic enzyme is involved as removal of the microsomes did not prevent abacavir metabolism in B-cells. This would tend to rule out CYP-mediated metabolism.

Aldehyde dehydrogenase 1 (ALDH1) is sensitive to disulfiram. Active aldehyde dehydrogenases are expressed in red blood cells but also lymphocytes and platelets (Helander and Tottmar, 1988). Given that the oxidation of carbovir is partly due to aldehyde dehydrogenase activity (Patanella and Walsh, 1992), it could therefore be predicted that abacavir is similarly metabolised. This enzyme is likely to catalyse the oxidation of abacavir aldehyde to carboxylic acid. In initial investigations disulfiram inhibited abacavir metabolism by

approximately 70%. Biological replicates are required in order to confirm this however.

In conclusion, a sensitive and reliable method for the quantification of abacavir and metabolites has been developed and applied to the *in vitro* oxidation of abacavir in human liver cytosol and immune cell preparations. In human liver cytosol abacavir is metabolised to three isomeric carboxylic acids via an aldehyde metabolite that can be trapped with methoxylamine. EBV-transformed B-cells have low-level metabolic activity but are capable of oxidising abacavir.

Chapter Five

Investigation of the mechanisms underlying activation of abacavir-specific T-cell clones isolated from HLA-B*57:01 positive healthy volunteers

5.1 Introduction

A number of hypotheses have been proposed to explain the ability of small molecules such as drugs to initiate an immune response against host cells. Drugs or drug metabolites may bind covalently to proteins before being taken up by specialised antigen-presenting cells. These haptenated proteins are then processed within the cell, and cleaved into peptide fragments which can then be presented to T-cells via MHC molecules (Park et al., 2001, Landsteiner and Jacobs, 1935). Alternatively the p-i concept suggests that the interaction between drug, T-cell receptor and MHC molecule can be non-covalent in nature and that direct stimulation of T-cells occurs, independent of cellular processing (Pichler, 2002). Both of these mechanisms highlight that the unique interaction between drug, T-cell receptor and MHC molecule is a key factor in the development of immune-mediated adverse reactions to drugs and as such, genetic associations involving specific HLA alleles may indicate why only small numbers of patients ultimately develop a reaction.

The generation of T-cell clones allows the study of these mechanisms *in vitro*. It must however be stressed that these investigations only reveal the mechanisms of activation *in vitro* and in particular the recall response. Very little is known about the initial priming of the response *in vivo*. These studies often indicate that multiple mechanisms of T-cell activation can occur simultaneously. In the case of sulfamethoxazole (SMX), distinct populations of T-cell clones can be generated that are either parent drug specific, metabolite-specific or cross-reactive (Castrejon et al., 2010). T-cell clones activated by the parent drug

respond rapidly (Zanni et al., 1998) and in the absence of antigen processing (Schnyder et al., 2000) (Castrejon et al., 2010). Washing antigen-presenting cells pulsed with SMX for 1h abrogates the T-cell response (Castrejon et al., 2010). Metabolite-specific T-cell clones in contrast, are activated rapidly by SMX-NO but require longer incubation with SMX. This mirrors the time required for the formation of irreversible protein adducts *in vitro* (Elsheikh et al., 2011). T-cell responses to SMX-NO are blocked by both fixation of antigen-presenting cells and addition of glutathione (Castrejon et al., 2010).

The immune-basis of abacavir hypersensitivity is well established. T-cells isolated from abacavir-hypersensitive patients secrete pro-inflammatory cytokines, including interferon-γ (Almeida et al., 2008) and TNF-α, (Martin et al., 2004) when stimulated *ex vivo.* Skin biopsies from inflamed skin of hypersensitive patients show a marked infiltration of $CD8^+$ T-cells, suggesting that tissue damage develops as a result of cytotoxic T-cell activity (Phillips et al., 2002). This is supported by the discovery that abacavir-specific $CD8^+$ T-cells were capable of killing target cells *in vitro* (Chessman et al., 2008). The mechanisms underlying T-cell activation by abacavir however are not yet fully understood. Key residues located within the F-pocket of HLA-B*57:01 (Ser116 and Asp114) are particularly important in antigen presentation, whether this be the parent drug or a drug-modified peptide (Chessman et al., 2008). This indicates that the interaction between drug, MHC molecule and TCR is highly specific. Analogues of abacavir were therefore synthesised inorder to examine cross-reactivity.

5.2 Aims

- Examine the structure-activity relationship of abacavir analogues at the HLA-TCR interface.
- Investigate the mechanisms of antigen presentation associated with T-cell activation by abacavir

5.3 Materials and Methods

5.3.1 Chemicals and reagents

Human AB serum was obtained from Innovative Research (Michigan, USA). Foetal bovine serum (FBS) was obtained from Invitrogen, Paisley, UK. Tetanus toxoid (TT) was bought from the Statens Serum Institut, Copenhagen, Denmark. Interferon-γ ELISpot kits including antibodies and substrate solution were purchased from Mabtech, Stockholm, Sweden. [^{3}H]-Thymidine was purchased from Moravek (California, USA).

Abacavir sulfate and carbovir were kind gifts from GlaxoSmithKline. Dihydro abacavir, abacavir enantiomers and deuterated abacavir were synthesised by Emma Yang (Chemistry department, University of Liverpool). All other chemicals and reagents were supplied by Sigma-Aldrich, Dorset, UK.

5.3.2 Cell culture medium

T-cell medium was comprised of RPMI 1640 supplemented with 10% human AB serum, 25mM HEPES, 1000U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-glutamine and 25μg/ml transferrin.

Antigen-presenting cell medium was comprised of RPMI 1640 supplemented with 10% Foetal bovine serum, 25mM HEPES, 1000U/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine.

5.3.3 T-cell cloning

T-cell clones used in this chapter were generated according to the methods described previously (see 3.3.5). Seventy-four T-cell clones were available for investigation (see Table 3.6 and Figure 3.14).

5.3.4 Chemical cross-reactivity

T-cell cross-reactivity was determined by both proliferation and ELISpot assays. For the proliferation assay, T-cell clones (1×10^5) were incubated with each of the compounds (carbovir, dihydro abacavir, deuterated abacavir and abacavir enantiomers; Figure 5.1)(1, 5, 10, 50 and 100μM) in the presence of irradiated autologous EBV-transformed B-cells for three days. [^{3}H]-Thymidine (0.5μCi/well) was added for the final 16h and proliferation determined by scintillation counting.

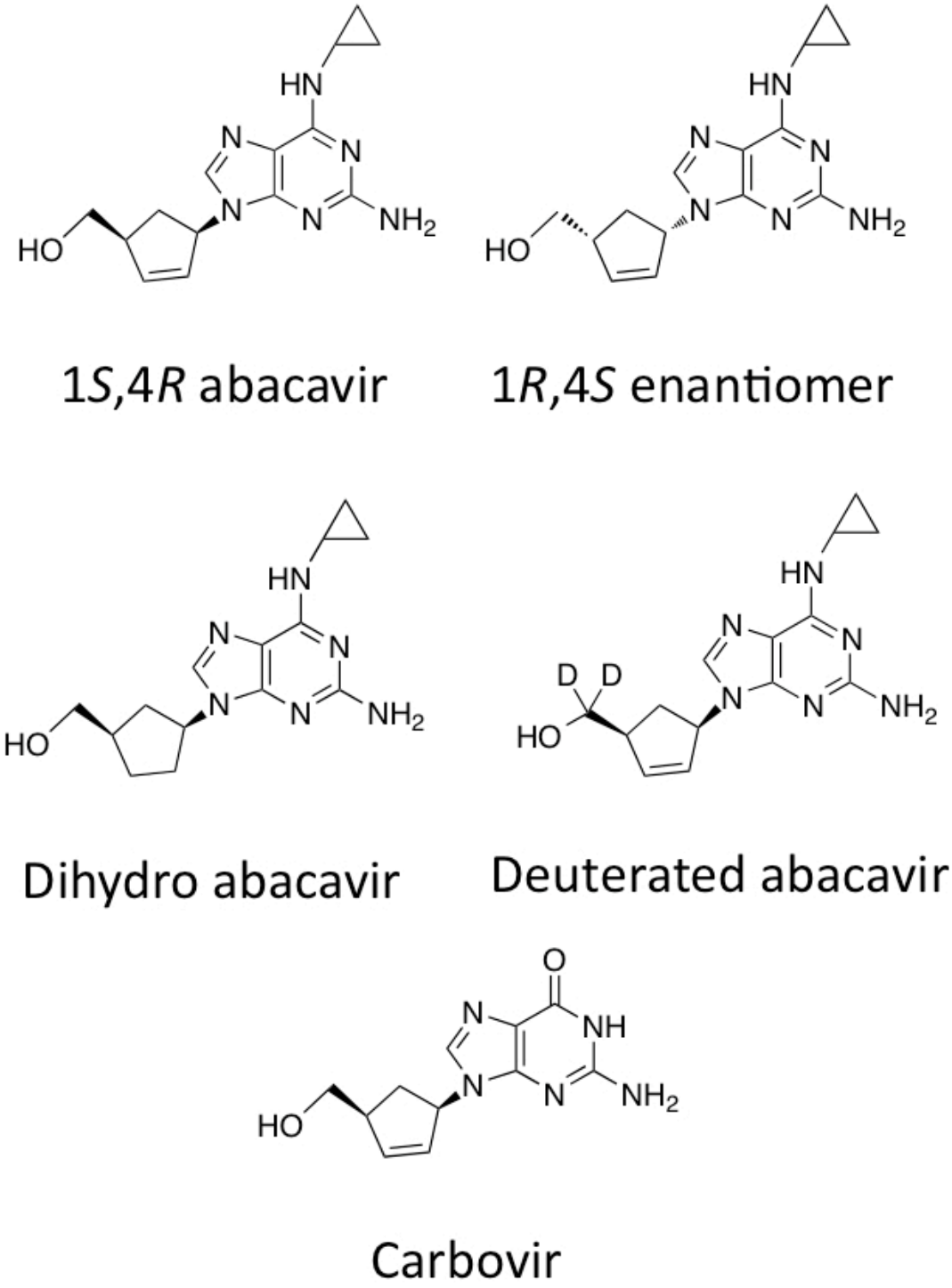

Figure 5.1 Abacavir analogues investigated in cross-reactivity experiments

For the ELISpot assay Multiscreen filter plates were coated with interferon-γ antibody (15μg/ml) overnight at 4˚C. Wells were washed with PBS (200μl) and blocked for 30 minutes with T-cell medium (200μl). T-cell clones were added to the plate along with irradiated autologous EBV-transformed B-cells and the compound of interest (1, 5, 10, 50 and 100μM). After 48h the plate was developed according to the manufacturers instructions. The wells were washed with PBS and incubated with secondary antibody (diluted 1:1000 in PBS containing 0.5% FBS) for 2 hours at room temperature. After washing with PBS, Streptavidin-ALP (diluted 1:1000 in PBS containing 0.5% FBS) was added to wells for one hour. Spots were visualised by the addition of BCIP/NBT substrate (100μl, 15 min). The plate was counted on an AID ELISpot reader (Cadama Medical, Stourbridge, UK) when thoroughly dried.

5.3.5 Mechanisms of antigen presentation

5.3.5.1 Fixation of antigen-presenting cells

The fixation assay is used to determine the role of intracellular processing in the T-cell response. Autologous EBV-transformed B-cells (2 x 10^6) were washed twice in HBSS and resuspended in 1ml HBSS. Glutaraldehyde (12.5μl, 2%) was then added and the cells agitated for 30 seconds. Glycine (1ml, 1M) was quickly added and cells were agitated for a further 45 seconds. The cells were resuspended in 10ml T-cell medium after extensive washing (3 times, T-cell medium). Glutaraldehyde-fixed EBV-transformed B-cells (1x 10^4, 50μl) were then added to T-cell clones (5 x 10^4) in the presence or absence of abacavir

(50μM). Cells were cultured for 3 days at 37˚C with [^{3}H]-thymidine added for the final 16h. Proliferation was determined by scintillation counting.

In order to determine the effect of glutaraldehyde fixation on cellular uptake of abacavir the intracellular accumulation of abacavir was investigated. EBV-transformed B-cells (fixed and unfixed)(20 x 10^6; 5ml) were incubated with 10μM abacavir for 1, 4 and 16 hours. At each time-point cells were harvested centrifuged and the supernatant removed. Cell pellets were resuspended in 5ml water and sonicated (4 x 10 sec). Deuterated abacavir (200nM; 50μl) was used as the internal standard. Acetonitrile (10 volumes) was added and precipitated proteins were removed by centrifugation. Samples were evaporated to dryness and resuspended in 50μl mobile phase (10mM ammounium acetate, pH 3.8) prior to LC-MS/MS analysis (see 4.3.2 for details of LC-MS/MS conditions).

5.3.5.2 Pulsing of antigen-presenting cells

The pulsing assay determines the importance of protein binding (either covalent or non-covalent) in the T-cell response. Autologous EBV-transformed B-cells (1 x 10^6) were incubated with abacavir for 1, 4 or 16 hours. Cells were washed three times in T-cell medium, counted and irradiated before adding to T-cell clones. No further drug was added to the incubations. In some instances EBV-transformed B-cells were fixed with glutaraldehyde prior to pulsing. The response was quantified by either proliferation or IFN-γ ELISpot as described previously.

5.4 Results

5.4.1 Chemical cross-reactivity

The specificity of T-cell clones was determined by both proliferation and IFN-γ ELISpot assays. The alternative enantiomer of abacavir (1*R*,4*S*) did not stimulate any of the clones tested to proliferate (Figure 5.2 A) or secrete cytokines (Figure 5.2B). Dihydro abacavir, which lacks a double bond on the cyclopentane ring, stimulated some clones to proliferate but only at the highest concentrations (50 and 100μM)(Figure 5.3A and B) and at a decreased magnitude compared to abacavir. Carbovir did not stimulate any of the clones tested (Figure 5.4). Deuterated abacavir stimulated clones to the same degree as 1S,4R abacavir (Figure 5.5).

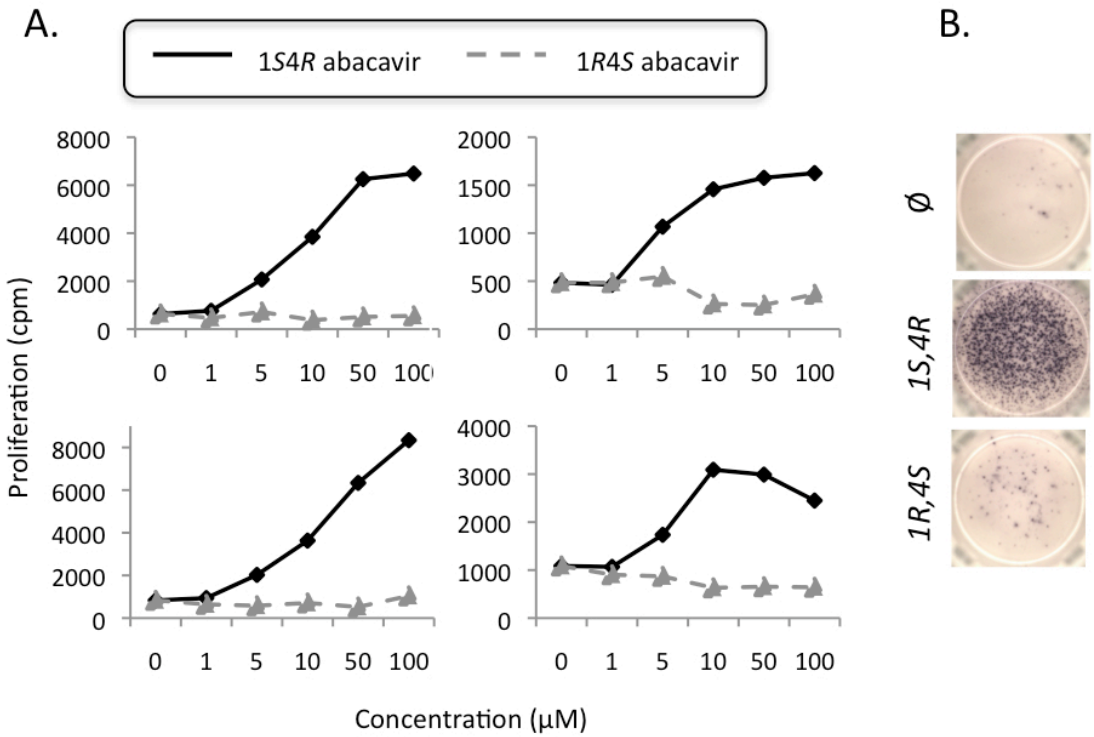

Figure 5.2 Cross-reactivity of four abacavir-specific T-cell clones to the alternative abacavir enantiomer (1*R*,4*S*) was determined by both A) proliferation and B) interferon-γ ELISpot.. ELISpot wells show secretion of IFN-γ at a 50μM dose of each compound. Clones were isolated from volunteer 024.

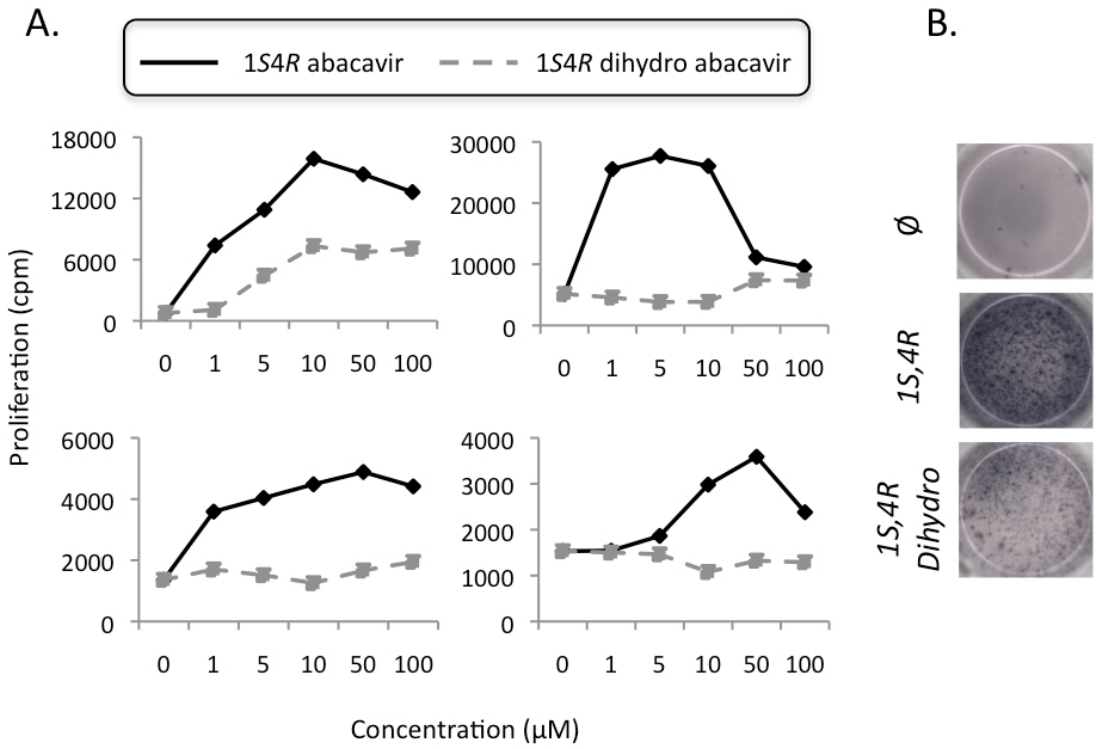

Figure 5.3 Cross-reactivity of four abacavir-specific T-cell clones to dihydro abacavir was determined by both A) proliferation and B) interferon-γ ELISpot. ELISpot wells show secretion of IFN-γ at a 50μM dose of each compound. Clones were isolated from volunteer 024.

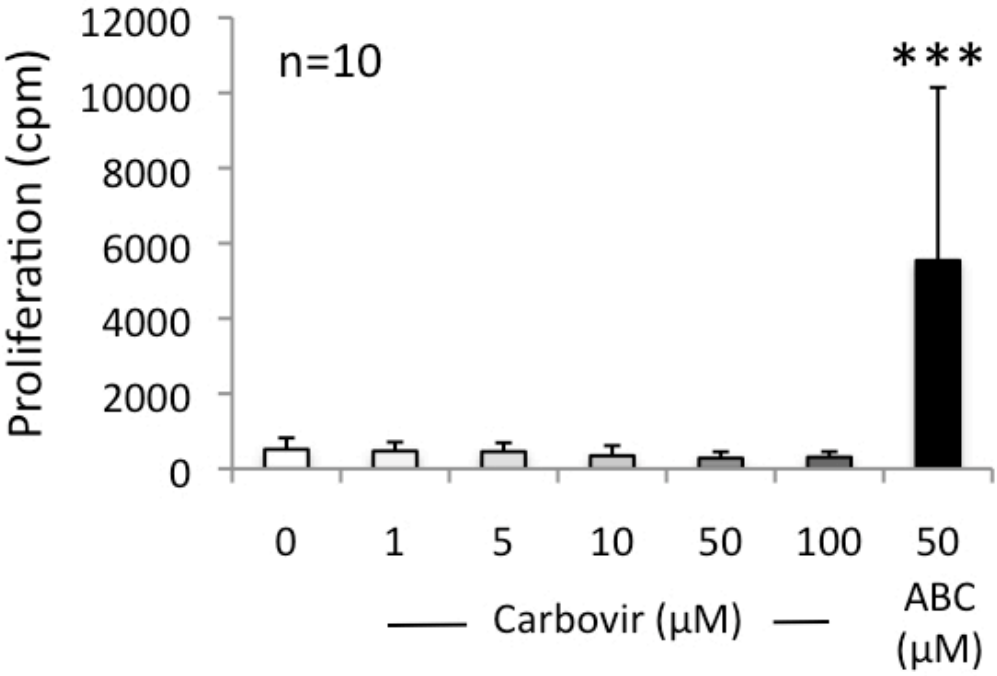

Figure 5.4 Cross-reactivity of ten abacavir-specific T-cell clones to carbovir was determined by proliferation assay. Abacavir (ABC; 50μM) served as a positive control (*** =p<0.001; one way ANOVA). Clones were isolated from volunteer 024.

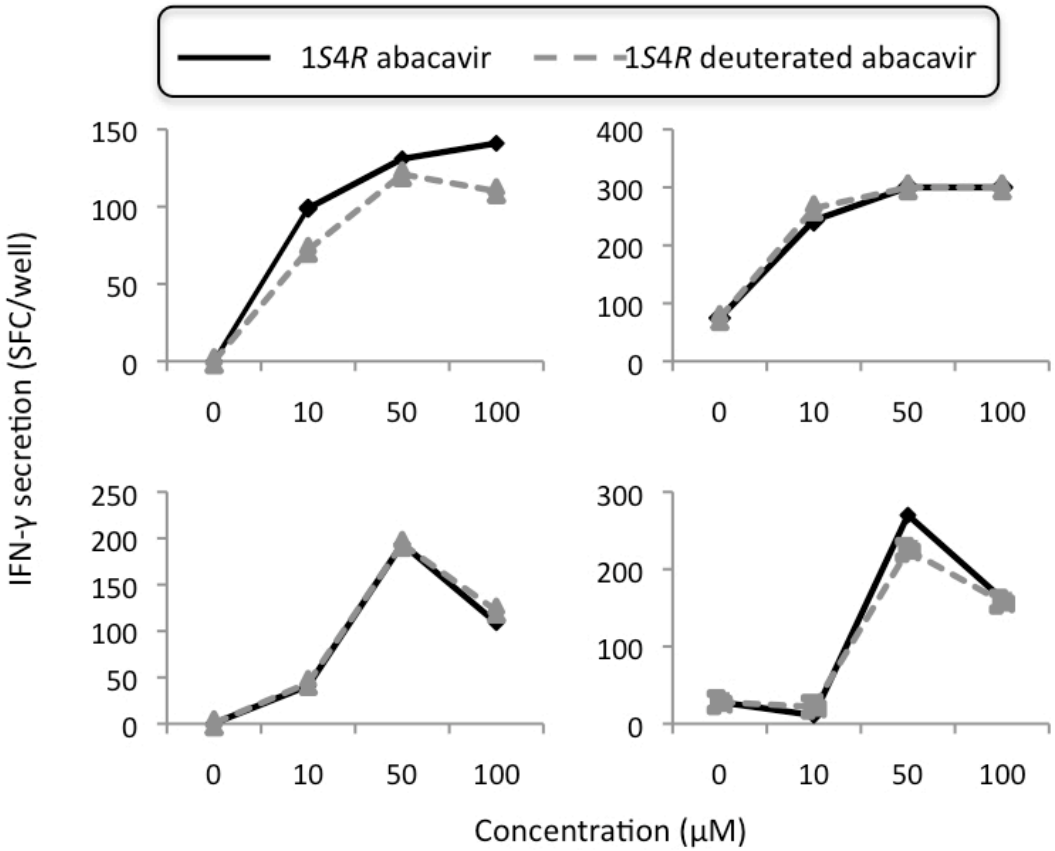

Figure 5.5 Cross-reactivity of four abacavir-specific T-cell clones to deuterated abacavir was determined by IFN-γ ELISpot. SFC= spot forming cell. Clones were isolated from volunteer 024.

5.4.2 Mechanisms of antigen presentation

Both processing dependent and independent clones were observed (Figure 5.6A and B). However given that MHC class I molecules are expressed by all nucleated cells, the possibility of self-presentation was also investigated by omitting the supplementary antigen-presenting cells. Abacavir stimulated 45% of the clones to proliferate and secrete IFN-γ directly, in the absence of irradiated EBV-transformed B-cells (Figure 5.7A). The remaining clones were activated only in the presence of transformed B-cells (Figure 5.7B); accordingly, these clones were used to probe mechanisms of drug antigen presentation

further. EBV-transformed B-cells pulsed with abacavir for 16h stimulated 10 out of 19 clones to proliferate and secrete IFN-γ. Exposure of abacavir to antigen-presenting cells for a shorter duration (1h) did not activate the clones (Figure 5.8). To confirm that the response of clones against abacavir-pulsed antigen-presenting cells was dependent on intracellular processes, transformed B-cells were fixed with glutaraldehyde prior to abacavir exposure. The T-cell response directed against abacavir pulsed antigen-presenting cells was blocked by fixation (Figure 5.9). The effect of glutaraldehyde fixation on abacavir uptake was determined by mass spectrometry. The intracellular concentration of abacavir was compared between fixed and unfixed EBV-transformed B-cells following incubation with abacavir for 1, 4 or 16 hours (Figure 5.10A). Abacavir was found to enter the cell rapidly and the intracellular concentration was maintained throughout the course of the incubation.

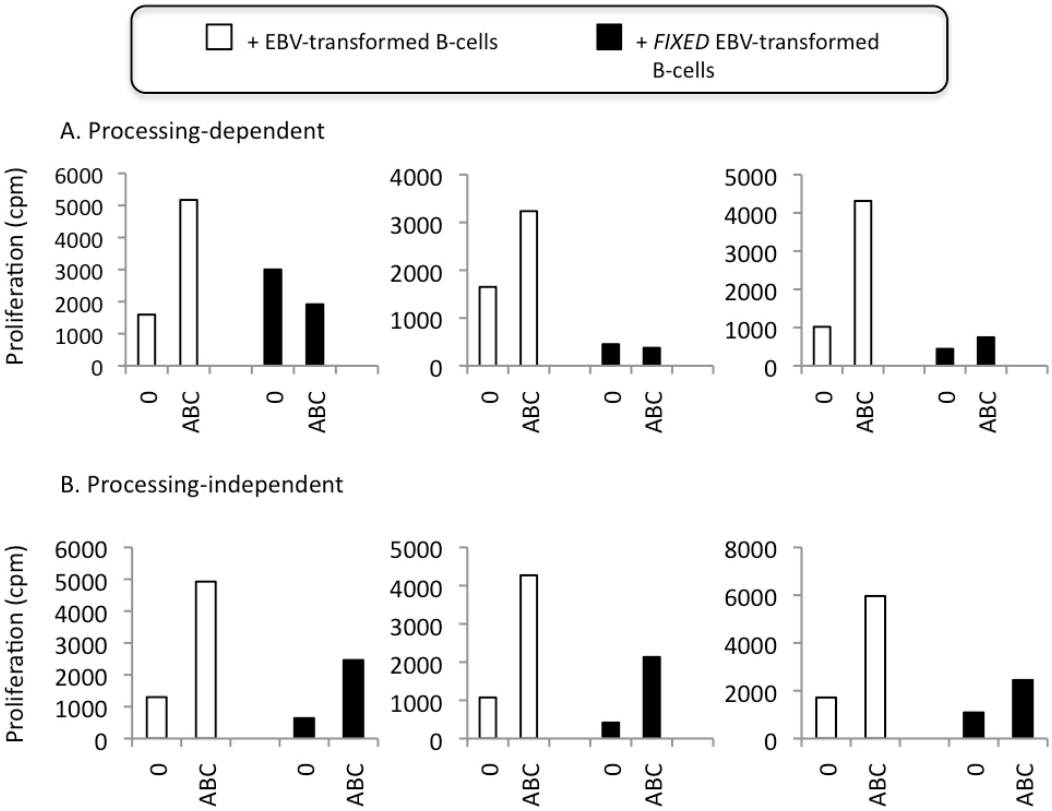

Figure 5.6 Importance of intracellular processing to abacavir-specific T-cell response. T-cell clones were incubated with irradiated autologous antigen-presenting cells (white bars) or antigen-presenting cells that had undergone glutaraldehyde fixation (black bars) and abacavir (50μM). Proliferation was determined by [^{3}H]-thymidine incorporation. Each graph represents an individual T-cell clone. Clones were isolated from volunteer 024.

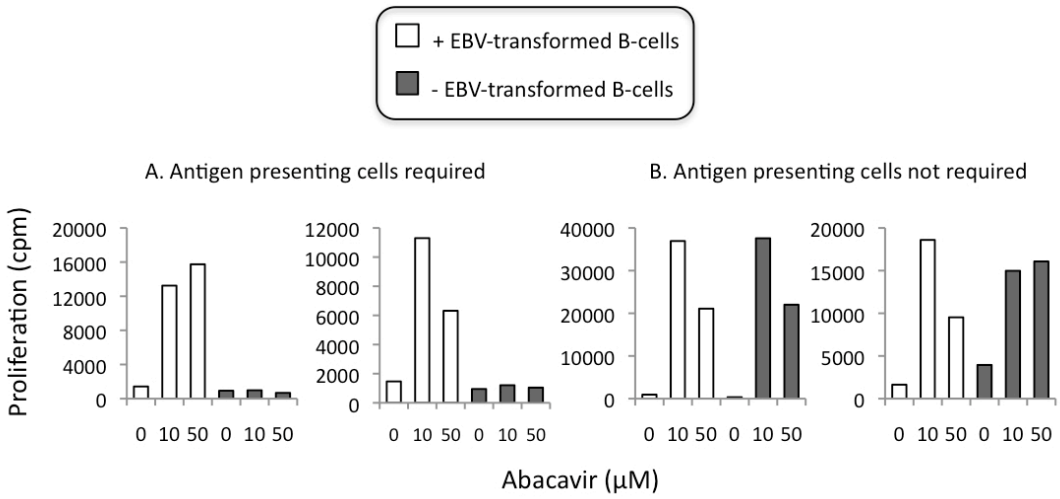

Figure 5.7 Abacavir-specific T-cell clones can be activated in the absence of supplementary antigen-presenting cells. T-cell clones were incubated either with (white bars) or without (grey bars) the addition of autologous EBV-transformed B-cells in the presence of abacavir. Proliferation was determined by [^{3}H]-thymidine incorporation. Each graph represents an individual T-cell clone. Clones were isolated from volunteer 024.

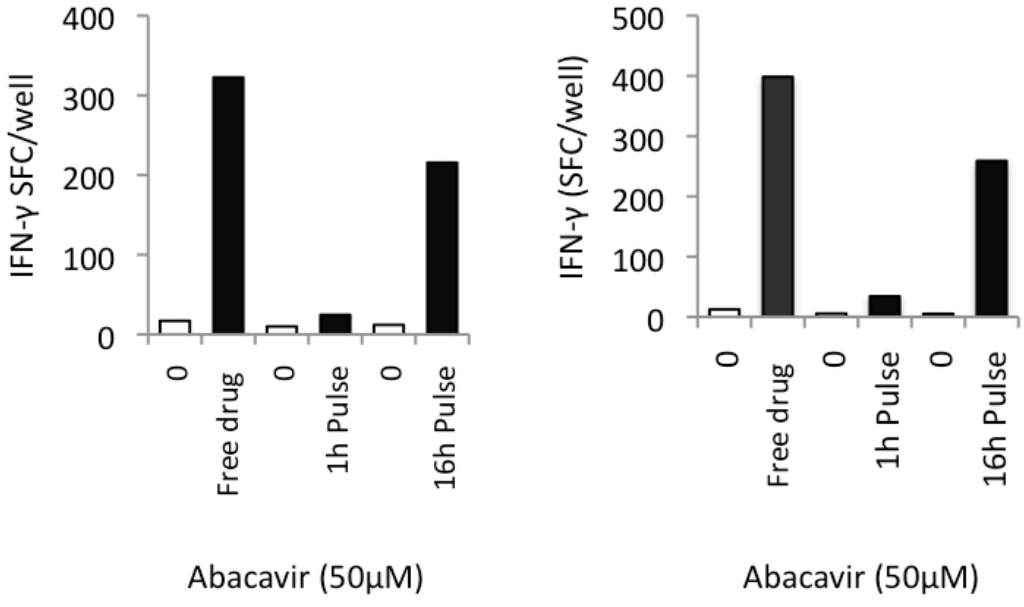

Figure 5.8 Time-dependency of abacavir-specific T-cell response. Autologous EBV-transformed B-cells were pulsed for 1 or 16 hours with abacavir (50µM) before adding to T-cell clones. T-cell response was quantified by IFN-γ ELISpot. Each graph represents an individual T-cell clone. SFC= spot forming cell. Free drug represents T-cell clones that were stimulated with abacavir continuously present in the well. Clones were isolated from volunteer 024.

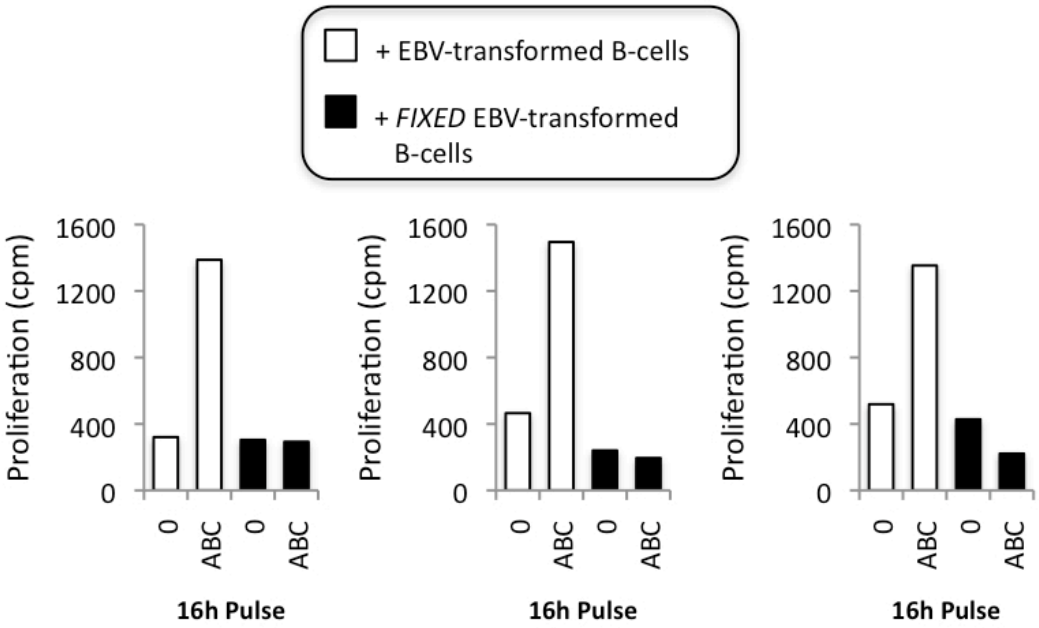

Figure 5.9 The T-cell response to pulsed antigen-presenting cells is dependent on the presence of functional antigen-presenting cells. Autologous EBV-transformed B-cells were pulsed for 16 hours with abacavir (ABC; 50μM) before adding to T-cell clones (white bars). The black bars represent EBV-transformed B-cells that had been subjected to fixation prior to pulsing with abacavir. Proliferation was determined by [^{3}H]-thymidine incorporation. Each graph represents an individual T-cell clone.

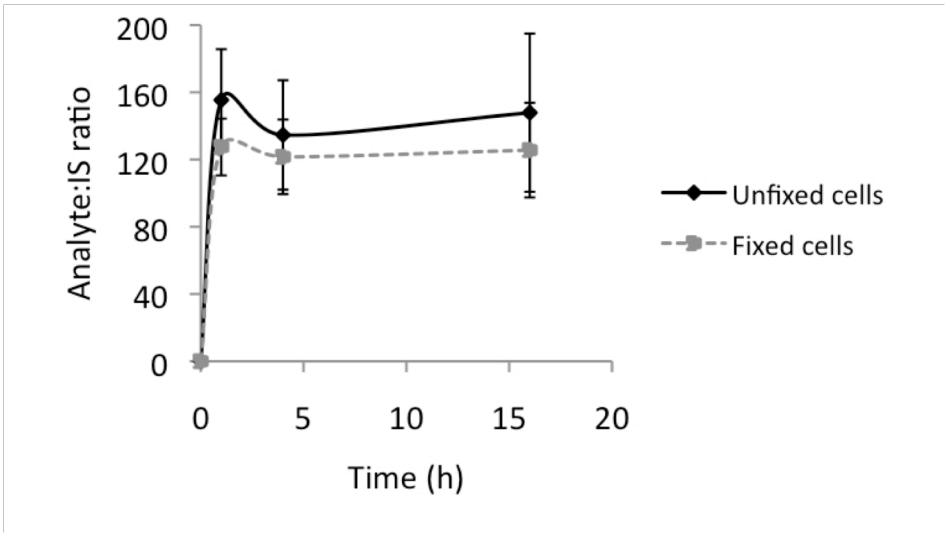

Figure 5.10 The time-course of abacavir uptake (A), Antigen-presenting cells were incubated with abacavir (10μM) for 1, 4 or 16h. Fixed cells were subjected to glutaraldehyde treatment

prior to incubation. Intracellular abacavir was measured by mass spectrometry. Data represent the mean ± SD of three independent experiments.

5.5 Discussion

In this chapter, abacavir-specific T-cell clones isolated from healthy volunteers expressing HLA-B*57:01 were subjected to a number of *in vitro* assays commonly used to determine chemical cross-reactivity and the mechanisms underlying T-cell activation.

In order for abacavir to initiate a T-cell response, it is possible that abacavir binds directly to HLA-B*57:01 or an HLA-B*57:01 binding peptide. Alternatively, abacavir metabolites could bind irreversibly to protein, forming abacavir-modified HLA-B*57:01 binding sequences that will be released through protein processing. With reference to the latter, the identity of proteins modified with abacavir metabolites, the nature of the binding interaction and the number of modifications needed to stimulate T-cells remains unresolved.

Abacavir analogues were synthesised in order to examine the nature of the interaction between the drug and HLA-B*57:01. Chessman et al. (2008) previously investigated the immunogenicity of carbovir, didanosine and guanosine in PBMCs isolated from hypersensitive patients and found that none of these compounds were able to stimulate abacavir-specific T-cells. All of these compounds lack the cyclopropyl ring moiety and hence this was implicated in the immunogenicity of abacavir. In this investigation the alternative enantiomer of abacavir did not stimulate any T-cell clones and dihydro abacavir was only

stimulatory in certain clones and at high concentrations. Previously dihydro abacavir has been shown to form fewer covalent adducts with human serum albumin than abacavir itself (Walsh et al., 2002). This could explain the reduced immunogenicity of dihydro abacavir. Chemical restriction of the abacavir-specific T-cell response could also be due to the altered stereochemistry of the molecules impacting on the direct interaction with HLA-B*57:01. Deuterated abacavir is metabolised more slowly than abacavir at least in liver preparations (see Chapter 4). The fact that it still stimulates clones at an equivalent level to abacavir itself may therefore be interesting. However it is thought that only a very small number of antigen molecules need to interact with TCRs in order for them to be stimulatory (Brower et al., 1994) and given that incubation times for the various assays can be up to 48 hours it is unclear whether the reduced rate of metabolism is likely to be important.

Omitting antigen-presenting cells from the assay revealed that some clones did not require the presence of supplementary antigen-presenting cells. This is consistent with the fact that MHC class I molecules are expressed on almost all nucleated cells. The role of antigen uptake and processing in the T-cell response is commonly determined by fixation of antigen-presenting cells (Schnyder et al., 1997). Glutaraldehyde cross-links proteins. Cells fixed in this way are unable to process antigens. Fixation does not alter MHC expression, presentation of peptides that have already been processed or the direct interaction of drugs with MHC molecules (Zanni et al., 1998, Shimonkevitz et al., 1983). The effect of fixation on drug uptake however has not been studied. Half of the clones tested in this investigation were activated in the presence of fixed antigen-presenting

cells. These clones are either able to self-present or are stimulated by the parent drug or a metabolite binding directly to an MHC-peptide complex at the cell surface. Vacant MHC molecules are not transported to the cell surface. Instead they are degraded in the cytosol (Hughes et al., 1997). Any cell surface interaction would therefore be with an occupied MHC molecule. This might suggest that any bonds formed would be between the drug and the bound peptide.

It could be hypothesised that glutaraldehyde fixation would impact on the cellular uptake of drugs through the disruption of transporters or the cell membrane. However, this was not found to be the case. This is probably due to the fact that abacavir is taken up passively (Mahony et al., 2004). Abacavir is a substrate for the efflux transporter P-glycoprotein (P-gp) (Shaik et al., 2007) which might suggest that fixation would lead to increased accumulation of abacavir were the transporter to be impaired. This was not observed. Abacavir was taken up rapidly by cells (both fixed and unfixed) and maintained at this concentration for up to 16 hours. This is in agreement with other studies in which abacavir can be detected within the cell following just a few minutes incubation (Mahony et al., 2004) .

Pulsing of antigen-presenting cells is used to determine the role of drug/metabolite binding to peptides or the MHC. Following 1 hour or overnight incubation with the drug, antigen-presenting cells are washed thoroughly prior to co-incubation with T-cells. Stable drug-MHC/peptide complexes are resistant to washing. This interaction does not have to be covalent in nature, indeed

multiple non-convalent bonds such as Van der Waals forces or coordinate bonds could be just as strong as a covalent bond. Pulsing for 1h was not sufficient to stimulate T-cell clones. As stated previously, abacavir is rapidly taken up by antigen-presenting cells meaning it would be available to bind to MHC molecules during this time. These could be vacant MHC molecules or MHC molecules already containing a peptide. In contrast, 10/19 T-cell clones were stimulated to proliferate and/or secrete interferon-γ by APCs pulsed with abacavir for 16 hours. The time-dependency of the APC pulsing T-cell response is difficult to reconcile with a direct interaction of abacavir with surface or endogenous MHC molecules, as abacavir is present within the cell at similar levels at both of these time-points. Interestingly, 16 hours is the time required for abacavir metabolites to be generated in APCs (see Chapter 4) However, in the absence of a specific inhibitor to block abacavir metabolism in immune cells, its role in the T-cell response is difficult to prove. Fixation of APCs prior to pulsing with abacavir indicates that functional APCs are required and that processing mechanisms are important in the pulsed response. This could be the processing of self-peptides or haptenated peptides.

Our findings must be discussed in the context of recent findings by a number of groups that have shown that abacavir binds to endogenous MHC molecules and alters the repertoire of peptides displayed on the cell surface (Illing et al., 2012a, Norcross et al., 2012, Ostrov et al., 2012) (discussed in Chapter 6). The processing of self-peptides is likely to be an important step in immune recognition by this mechanism.

The isolation of abacavir-specific T-cell clones from HLA-B*57:01 positive healthy volunteers provided a medium through which T-cell phenotype and functionality could be investigated in more detail. T-cell clones are highly specific and seemingly subtle changes in the abacavir molecule can impact on T-cell receptor recognition. Mechanisms of activation are diverse and may be clone-dependent. It is currently difficult to differentiate between metabolite-specific and peptide-specific responses but it is likely that *in vivo* multiple mechanisms occur simultaneously.

Chapter Six
Final discussion

Adverse drug reactions (ADRs) are a serious health problem and a frequent cause of drug withdrawals. Type B off-target reactions are particularly difficult to predict and affect only a small number of susceptible individuals. Many of these reactions have an immune aetiology.

Most experimental work to date has focussed on the characterisation of cells isolated from hypersensitive patents and in this way much can be learned about the cellular phenotype and cytokine profile of drug-reactive cells. Drug-specific cytotoxic T-cells can often be identified and this may give an indication of the cause of cellular damage. Multiple mechanisms of T-cell activation have been hypothesised (see 1.5) and evidence supporting each of these can be inferred from the characteristics of T-cell clones isolated from hypersensitive patients. It is still difficult to interpret how this relates to the *in vivo* situation however as experimental findings are based on a recall response. In addition to traditional proliferative responses measured in the standard LTT assay, cytokine secretion as determined by ELISpot is increasingly used to characterise drug hypersensitivity (Rozieres et al., 2009). This is particularly useful where $CD8^+$ T-cells are involved in the reaction and may indeed improve the sensitivity of *in vitro* assays.

The emergence of a variety of highly specific HLA associations has however both provided a means through which to predict at-risk patients and also shed further light on the underlying mechanisms. A diverse array of adverse

reactions have now been reported to be associated with possession of a specific HLA molecule and both skin and liver reactions are represented. Given that specific HLA molecules may have a functional role in antigen presentation, future work to understand the mechanisms involved must be performed with cells from a known genetic background. A cohort of 400 volunteers was therefore established in order to investigate these associations *in vitro*.

The association between HLA-B*57:01 and abacavir hypersensitivity represents a prime example of how pharmacogenomics can be effectively translated into the clinic. Genotyping prior to prescription has been introduced on a global scale, effectively reducing the incidence of the reaction (Hughes et al., 2004). A total of 26 individuals expressing HLA-B*57:01 were identified in our cohort and cells from these volunteers were used in *in vitro* assays.

Drug-specific T-cells are often implicated in the pathogenesis of drug hypersensitivity reactions. An important breakthrough however came with the detection of drug-reactive T-cells isolated from healthy volunteers that have never been exposed to abacavir (Chessman et al., 2008). A similar phenomenon has also recently been observed with carbamazepine and HLA-B*15:02 (Ko et al., 2011). Our attempts however to replicate these findings were largely unsuccessful (Chapter 3). This may be due to technical difficulties or the alternative readouts examined. Abacavir-specific T-cell clones however could be generated from all HLA-B*57:01 volunteers tested. This may indicate that the number of drug-reactive cells was too low to detect by either proliferation or

cytokine ELISpot directly. It was only through analysing single cell populations that robust responses were observed.

Traditionally T-cell responses detected by either LTT or ELISpot assay have been attributed to the expansion of a memory cell population following the initial priming of the immune response *in vivo*. Could these cells therefore be from a cross-reactive population? The cross-reacting antigen would need to be prevalent in a number of different populations and uniquely restricted by HLA-B*57:01. It may however explain why only a proportion of patients expressing HLA-B*57:01 develop a hypersensitivity reaction when exposed to abacavir; perhaps they must also have previously encountered a cross-reacting viral peptide. Interestingly, HLA-B*57:01 restricted $CD8^+$ T-cells are more cross-reactive to point mutants of viral peptides than T-cells restricted by other HLA alleles (Kosmrlj et al., 2010). This is thought to be because HLA-B*57:01 binds fewer self-peptides than other alleles and therefore encounters fewer peptide-TCR sequences during thymic development. Precursor frequencies are also predicted to be higher in a HLA-B*57:01 restricted repertoire (Kosmrlj et al., 2010). Possession of the HLA-B*57:01 allele is protective against HIV infection (Migueles et al., 2000), possibly because mutations in the HIV virus do not impact upon immune recognition.

Nevertheless, it was found that the abacavir-specific T-cell clones that were isolated in the present study were highly specific in a chemical sense and not activated by various analogues of abacavir (Chapter 5). This may indicate that there is more to the interaction than a simple fit of the molecule in a specific

TCR. The specificity appears to reside at least in part, within the HLA-B*57:01 molecule itself, as previously reported (Chessman et al., 2008).

Chessman et al. (2008) previously investigated the immunogenicity of carbovir, didanosine and guanosine in PBMCs isolated from hypersensitive patients and found that none of these compounds were able to stimulate abacavir-specific T-cells. All of these compounds lack the cyclopropyl ring moiety and hence this was implicated in the immunogenicity of abacavir. In this investigation the alternative enantiomer of abacavir did not stimulate any T-cell clones and dihydro abacavir was only stimulatory in certain clones and at high concentrations. Previously dihydro abacavir has been shown to form fewer covalent adducts with human serum albumin than abacavir itself (Walsh et al., 2002). This could explain the reduced immunogenicity of dihydro abacavir. Chemical restriction of the abacavir-specific T-cell response could also be due to the altered stereochemistry of the molecules impacting on the direct interaction with HLA-B*57:01. Deuterated abacavir is metabolised more slowly than abacavir at least in liver preparations (see Chapter 4). The fact that it still stimulates clones at an equivalent level to abacavir itself may therefore relate to the direct (metabolism independent) activation of T-cells. Alternatively, the levels of metabolism seen with deuterated abacavir might be sufficient to activate the T-cell response.

Alongside the T-cell assays, metabolic investigations were also performed. Chemically reactive metabolites are often implicated in drug hypersensitivity reactions. In order to make any conclusions regarding the mechanisms of T-cell activation it is therefore important to understand which chemical entities the

cells are exposed to. The oxidation of abacavir to a stable carboxylic acid metabolite proceeds via a reactive aldehyde (Walsh et al., 2002). This has been found to covalently bind to proteins both *in vitro* and *in vivo* (Charneira et al., 2011, Charneira et al., 2012). Whether these modified proteins are immunogenic is unclear.

In this investigation EBV-transformed B-cells were found to be metabolically active. This is important as these cells represent the only source of metabolising enzymes in the *in vitro* assays. Metabolites were formed in low amounts; nonetheless this indicates that cells are exposed to the reactive aldehyde during *in vitro* assays. Of course the major site of metabolism *in vivo* is the liver. It is difficult to suggest therefore what contribution abacavir metabolism plays *in vivo* versus *in vitro*. Reactive metabolites formed hepatically are unlikely to reach distant sites unchanged. Instead they are likely to bind to proteins in the vicinity in which they are formed. These modified proteins could then potentially circulate in the blood to other sites. However, if that were the case it is difficult to conceive why certain organs are targeted.

The major chemical entity present in our *in vitro* assays is however the parent drug. There have been considerable advances with regard to understanding how abacavir can interact with HLA-B*57:01 directly. A number of groups have all independently proposed a novel mechanism of drug interaction with HLA molecules (Ostrov et al., 2012, Norcross et al., 2012, Illing et al., 2012a). The central feature of each of these studies is that T-cell activation occurs independently of either drug bioactivation or hapten formation. Due to the

groundbreaking nature of these findings each of the papers will be discussed here.

*"Abacavir induces loading of novel self-peptides into HLA-B*57:01: an autoimmune model for HLA-associated drug hypersensitivity"* (Norcross et al. 2012)

In this investigation the authors attempted to define the influence of abacavir on peptide binding to HLA-B*57:01. Firstly, the authors determined the effect of abacavir on the strength of peptide binding to soluble HLA-B*57:01 molecules (sHLA-B*57:01). A fluorescently labelled self-peptide was incubated with sHLA-B*57:01 in the presence and absence of abacavir. Abacavir enhanced the binding of the peptide in a concentration-dependent manner. Neither didanosine (another NRTI) nor flucloxacillin (an antibiotic causing DILI in patients expressing HLA-B*57:01) had this effect. The authors suggest that this may represent a physiological interaction as the increase in peptide affinity was observed at doses as low as 10μM. Soluble HLA-B*57:01 molecules were then cultured in the presence or absence of abacavir and the bound peptides were analysed by mass spectrometry. Peptides common to both treated and untreated samples contained the expected HLA-B*57:01-binding P2 and C terminal (PΩ) anchor residues. These included alanine, threonine or serine at P2 and tryptophan, tyrosine or phenylalanine at PΩ. Following abacavir treatment, eluted peptides showed an increased preference for isoleucine or leucine at PΩ. No changes to the P2 residue were observed. Next, the authors investigated whether these peptides would only bind to sHLA-B*57:01 in the

presence of the drug. This was not the case. It is unclear however whether this could have been due to the use of soluble HLA molecules. The authors also suggested that abacavir metabolites might bind in a similar way to abacavir itself and hence have a similar effect.

"Immune self-reactivity triggered by drug-modified HLA-peptide repertoire" (Illing et al. 2012)

In this paper, the ability of a number of different HLA alleles to present abacavir to T-cells was assessed. In addition to alleles previously investigated (Chessman et al., 2008) the authors looked at HLA-B*57:11 which contains Ile94Thr, Ile95Leu and Val97Trp mutations located within the C/E pocket. This allele was not able to present abacavir to T-cells, further highlighting the importance of specific amino acid residues located within the binding groove of the HLA molecule.

The effect of abacavir treatment on the peptides eluted from cell lines expressing HLA-B*57:01 and HLA-B*57:03 was then investigated. HPLC fractions of peptides eluted from cells expressing HLA-B*57:01 but not HLA-B*57:03 contained unmodified abacavir, indicating that the drug was associated with the eluted peptides. No metabolites were observed, however as we have seen, in some cell types metabolites are formed in very low amounts that may be below the quantitative limit of the assay. Following drug treatment the characteristics of peptides eluted from cells expressing HLA-B*57:01 was

altered. Peptides displayed an increased preference for isoleucine or leucine at PΩ. No effect on the PΩ residue was observed in peptides eluted from cells expressing HLA-B*57:03 or HLA-B*58:01 following drug treatment.

In order to determine the biological importance of these observations, the authors generated T-cell lines from HLA-B*57:01 positive volunteers. TAP-deficient antigen-presenting cells were only able to induce a T-cell response when endogenous peptides were supplied in addition to abacavir. Abacavir alone was not stimulatory. This indicates the importance of antigen processing. The authors however did not mention whether the peptides alone i.e. in the absence of abacavir, were stimulatory. This peptide-specific response led the authors to investigate to TCR-Vβ repertoire of responding cells. Because T-cells were exposed to a number of different peptide epitopes it could be expected that TCR-Vβ useage would be polyclonal. This was indeed the case and is in agreement with data obtained from T-cell clones, presented in Chapter 3.

A key aspect of this work was the elucidation of a crystal structure of a self-peptide bound to HLA-B*57:01 in the presence of abacavir. This structure revealed that residues previously identified as being crucial for the presentation of abacavir to T-cells formed direct interactions with abacavir. In particular, Ser116 and Asp114 were found to form hydrogen bonds with the abacavir molecule. Extensive bonds were identified between abacavir and HLA-B*57:01, yet relatively few contacts were made between abacavir and the bound peptide.

The authors also attempted to apply these findings to the interaction of carbamazepine and HLA-B*15:02. Fewer alterations in peptide sequence were observed and these were confined to non-anchor positions. The authors however suggest that the presence of altered peptides indicates that the model is applicable to multiple forms of drug hypersensitivity.

"Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire" (Ostrov et al. 2012)

Prior to performing cellular assays, the authors utilised computer modelling techniques based on peptide libraries to screen HLA-B*57:01 binding peptides and predict how abacavir could influence peptide binding. This model predicted that the affinity of peptides containing a valine, alanine or isoleucine PΩ residue would be increased in the presence of abacavir. This is slightly different to the findings of the previous papers. Two peptides were then synthesised which differed only at the PΩ residue. These peptides were termed pep-V (containing a valine residue) and pep-W (containing a tryptophan residue). Based on the *in silico* modelling it was expected that pep-V would only bind to HLA-B*57:01 in the presence of abacavir. Indeed the binding affinity of pep-V increased in a concentration dependent manner. Molecular modelling suggested a potential binding site for abacavir located within the F pocket of HLA-B*57:01 close to Ser116. In order to investigate this further, a crystal structure of HLA-B*57:01 and pep-V in the presence of abacavir was solved. In agreement with the previous study, abacavir was found to make extensive contacts with HLA-

B*57:01 and fewer contacts with the bound peptide. Again, hydrogen bonds between abacavir and Ser116 and Asp114 were proposed. Peptides eluted from cell lines expressing HLA-B*57:01 largely matched those predicted by the peptide library. Peptides with valine and isolecine residues at the PΩ position were increased, whereas peptides with tryptophan or phenylanine were disfavoured. No peptides were eluted containing a PΩ alanine, though the authors explain that this is due to the antigen processing pathway which disfavours an alanine in this position.

The final part of the work utilised PBMCs isolated from abacavir hypersensitive individuals. PBMCs were incubated with various combinations of altered peptides and a high concentration of abacavir (100μg/ml) for 15 minutes. Cells were then washed and incubated in a coated ELISpot plate overnight. Cytokine secretion was increased in the presence of abacavir and the peptides compared to either abacavir or peptides alone. These responses however were considerably lower than those observed when PBMCs were incubated with a lower concentration of abacavir overnight.

Taken together these three papers suggest a novel mechanism of drug interaction with HLA-B*57:01. This can be simplified as shown in Figure 6.1. Abacavir binds to key residues located within the binding groove of HLA-B*57:01 altering the peptide specificity of the molecule. An immune response is initiated due to the recognition of a self-peptide that would not ordinarily be presented in the context of HLA-B*57:01.

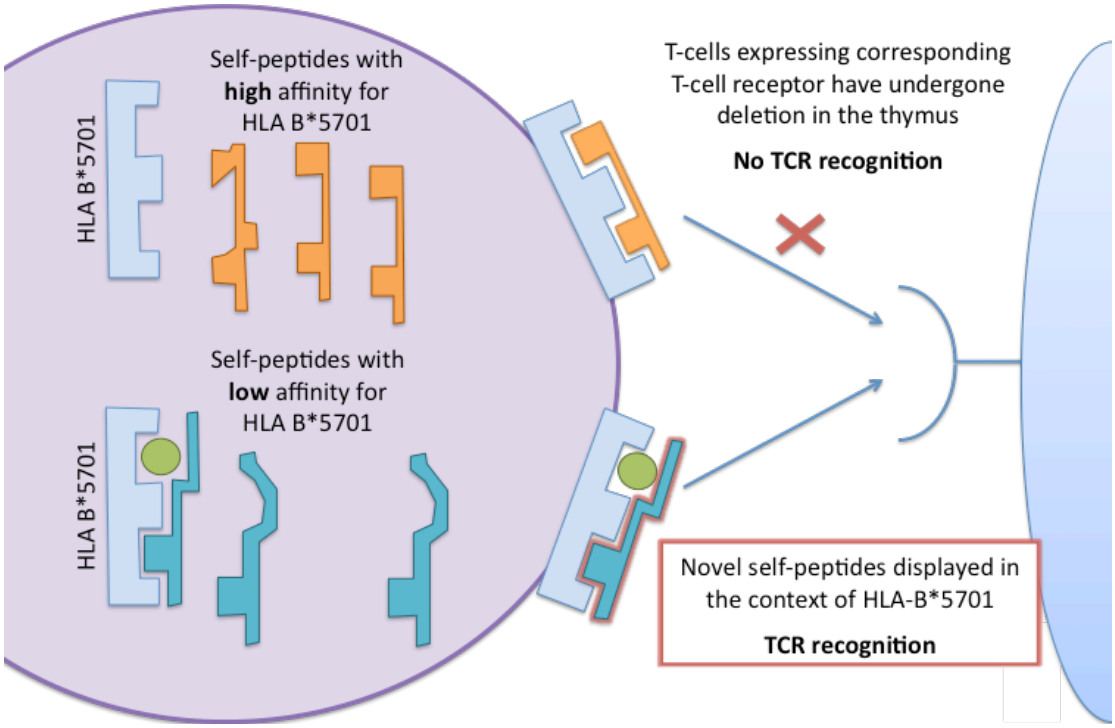

Figure 6.1 The altered peptide repertoire model. A non-covalent association of abacavir and HLA-B*57:01 leads to the loading of an altered set of peptides.

Our data would fit with these findings, at least for some clones. Where antigen processing is important in the T-cell response this could represent the involvement of endogenous self-peptides as described above or haptenated proteins formed following drug metabolism. It is unclear currently how to differentiate between these mechanisms *in vitro* as an altered self-peptide binding to HLA-B*57:01 in the presence of abacavir could be mimicking a peptide modified covalently with the drug or indeed *vice versa*.

Other clones however were activated even when antigen-presenting cells fixed with glutaraldehyde were used. This may be more in line with recent findings from Adam et al. (2012). A population of T-cell clones isolated from HLA-B*57:01 positive healthy volunteers was identified that could be activated

extremely rapidly (<200s) following drug treatment. Clearly, the speed of this activation would not allow any role for metabolism or processing of haptenated proteins.

The use of abacavir analogues has highlighted the chemical restriction of the abacavir-specific T-cell response. Molecular modelling techniques could be utilised in order to explore whether this restriction is based on the direct interaction of each of these compounds with HLA-B*57:01. *In silico* modelling could also be used to identify chemical structures that do not interact with HLA-B*57:01 with such high affinity. In collaboration with The Department of Chemistry at the University of Liverpool we are currently adopting this approach. Novel analogues of abacavir will be synthesized based on their interaction (or lack of) with HLA-B*57:01 and used in (1) T-cell assays to measure immunogenicity, (2) metabolic screens and (3) functional assays to measure anti-viral activity.

The field of drug hypersensitivity research has advanced greatly over the past four years. HLA associations have been observed for many adverse drug reactions and evidence is building that some of these associations may have a functional consequence (Chessman et al., 2008, Ko et al., 2011). As yet however few of these associations have been translated into a clinical setting (Phillips and Mallal, 2009). Further work is required in order to establish whether the functional relevance of HLA associations is applicable to other types of drug hypersensitivity or whether current findings are unusual to the drugs investigated so far. The strength of the association may also be an important

factor and weak associations with highly prevalent alleles may not yield such impressive results.

In addition, emerging evidence for the involvement of T-cells in DILI is a significant breakthrough (Monshi et al., 2013). Flucloxacillin-specific T-cell clones were generated from 5/6 patients investigated in the study, enabling phenotypic and mechanistic investigations to be performed. A possible explanation for the liver-specific effects of flucloxacillin may also have been uncovered, in that T-cell clones were found to express a variety of chemokine receptors (e.g CCR4 and CCR9) which are associated with T-cell migration to the liver. Furthermore T-cell clones were also generated from healthy volunteers expressing the relevant risk allele (HLA-B*57:01). In contrast to work described with both abacavir and carbamazepine, this was dependent on including an initial priming stage using autologous dendritic cells as antigen-presenting cells. The increased antigen-presenting capability of dendritic cells appears to give the method greater sensitivity and may therefore allow the investigation of other drugs associated with DILI (Faulkner et al., 2012).

Abacavir hypersensitivity represents an area in which novel methodologies have been applied to interrogate the interaction between drugs, proteins and cells. T-cell clones isolated from volunteers expressing HLA-B*57:01 have proven to be an incredibly useful tool in examining the mechanisms of antigen presentation and cytotoxicity relevant to the drug reaction. The interaction between abacavir and the HLA molecule is highly specific and seemingly minor alterations to both the drug molecule and the amino acids within the HLA

binding groove can have a significant impact on TCR recognition. Both processing-dependent and -independent models of antigen presentation have been reported, further indicating that the interaction is incredibly complex and cannot be explained by a single overriding mechanism. However, these studies and ours are limited in that they only describe the *in vitro* situation. It is currently unclear how these mechanisms are likely to interact *in vivo.* Additionally, the role of abacavir metabolism in the formation of antigens may be under-estimated in *in vitro* models that do not incorporate a significant source of metabolising enzymes. In order to further understand the contribution that drug metabolism plays in the generation of T-cell antigens it may therefore be useful to examine adduct formation in patients undergoing abacavir therapy. Continued work in this area may however lead to the improved design and development of drugs for the future.

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Appendix

Tables A1-A5 The frequency of HLA alleles among volunteers recruited to the cohort established in Chapter 2

Tables A6-A16 Gene expression data obtained from the NanoString analysis of HLA-B*57:01 positive and negative cells after treatment with abacavir, flucloxacillin or tetanus toxoid. Data signify the averaged counts from 3 volunteers in each group.

Table A.1 Distribution of HLA-A alleles in the HLA-typed cohort n=385

HLA-A				
Allele	**AF**	**GF (%)**	**Homozygotes**	**Heterozygotes**
A*010103	0.001	0.3	0	1
A*0101g	0.162	34.8	9	125
A*0106	0.001	0.3	0	1
A*020118	0.001	0.3	0	1
A*0201g	0.199	45.7	23	153
A*0202	0.005	1.0	0	4
A*0205	0.005	1.0	0	4
A*0205g	0.005	1.0	0	4
A*0206g	0.004	0.8	0	3
A*0207g	0.004	0.8	0	3
A*0211g	0.004	0.8	0	3
A*022001	0.001	0.3	0	1
A*0222g	0.001	0.3	0	1
A*0224	0.001	0.3	0	1
A*030103	0.001	0.3	0	1
A*0301g	0.108	23.1	6	83
A*0302	0.003	0.5	0	2
A*1101g	0.073	16.1	6	56
A*1102	0.003	0.5	0	2
A*2301g	0.018	3.6	0	14
A*2402g	0.084	16.9	0	65
A*2403g	0.001	0.3	0	1
A*2410	0.001	0.3	0	1
A*250101	0.008	1.6	0	6
A*2501g	0.012	2.3	0	9
A*2601g	0.026	5.2	0	20
A*2901g	0.004	0.8	0	3
A*290201	0.027	5.5	0	21
A*3001g	0.005	1.0	0	4
A*3002	0.008	1.6	0	6
A*3101g	0.030	6.0	0	23
A*3201	0.025	4.9	0	19
A*3301	0.001	0.3	0	1
A*330101	0.003	0.5	0	2
A*3303g	0.010	2.3	1	8
A*3601	0.001	0.3	0	1
A*6601	0.001	0.3	0	1
A*6601g	0.003	0.5	0	2
A*6801	0.005	1.0	0	4
A*6801g	0.016	3.1	0	12
A*6802	0.001	0.3	0	1
A*680201	0.005	1.3	1	4
A*7403	0.001	0.3	0	1

Table A.2 Distribution of HLA-B alleles in the HLA-typed cohort n=385

HLA-B				
Allele	**AF**	**GF (%)**	**Homozygotes**	**Heterozygotes**
B*0702g	0.134	24.4	9	85
B*070503	0.001	0.3	0	1
B*0705g	0.006	1.3	0	5
B*080104	0.004	0.8	0	3
B*0801g	0.122	23.6	3	88
B*1301	0.003	0.5	0	2
B*130101	0.001	0.3	0	1
B*1302	0.004	0.8	0	3
B*130201	0.003	0.5	0	2
B*1401	0.009	1.8	0	7
B*140201	0.023	4.7	0	18
B*150102	0.003	0.5	0	2
B*1501g	0.053	10.4	1	39
B*1502	0.010	1.8	1	6
B*1503g	0.003	0.5	0	2
B*1505	0.001	0.3	0	1
B*1507	0.003	0.5	0	2
B*151101	0.001	0.3	0	1
B*1513	0.003	0.5	0	2
B*1516	0.001	0.3	0	1
B*1517	0.004	0.8	0	3
B*1518	0.005	1.0	0	4
B*152501	0.003	0.5	0	2
B*1801g	0.035	7.0	0	27
B*1802	0.001	0.3	0	1
B*2702	0.003	0.5	0	2
B*2705g	0.044	8.6	1	32
B*2707	0.001	0.3	0	1
B*3501g	0.030	6.0	0	23
B*350201	0.004	0.8	0	3
B*3503g	0.016	3.1	0	12
B*3505	0.001	0.3	0	1
B*350801	0.003	0.5	0	2
B*370101	0.021	4.2	0	16
B*380101	0.009	1.8	0	7
B*3901g	0.006	1.3	0	5
B*3905	0.001	0.3	0	1
B*390602	0.001	0.3	0	1
B*400103	0.001	0.3	0	1
B*4001g	0.055	10.4	2	38
B*4002g	0.006	1.3	0	5
B*4006	0.010	2.1	0	8
B*400601	0.005	1.0	0	4
B*4101	0.003	0.5	0	2
B*410201	0.001	0.3	0	1
B*4402g	0.084	16.1	3	59

HLA-B continued				
Allele	**AF**	**GF (%)**	**Homozygotes**	**Heterozygotes**
B*4403	0.047	9.1	1	34
B*440302	0.009	1.8	0	7
B*4404	0.001	0.3	0	1
B*4405	0.001	0.3	0	1
B*4501g	0.004	0.8	0	3
B*460101	0.001	0.3	0	1
B*4601g	0.003	0.5	0	2
B*4701	0.003	0.5	0	2
B*470101	0.001	0.3	0	1
B*4801g	0.001	0.3	0	1
B*4901	0.003	0.5	0	2
B*490101	0.004	0.8	0	3
B*5001	0.016	3.1	0	12
B*5002	0.001	0.3	0	1
B*5101g	0.048	9.4	1	35
B*520102	0.001	0.3	0	1
B*5201g	0.019	3.6	1	13
B*530101	0.009	1.8	0	7
B*5401	0.001	0.3	0	1
B*5401g	0.001	0.3	0	1
B*5501	0.022	4.4	0	17
B*550201	0.003	0.5	0	2
B*5601	0.004	0.8	0	3
B*5601g	0.005	1.0	0	4
B*570101	0.035	6.8	1	25
B*570301	0.003	0.5	0	2
B*5801g	0.008	1.6	0	6
B*7301	0.001	0.3	0	1
B*8101g	0.001	0.3	0	1

Table A.3 Distribution of HLA-C alleles in the HLA-typed cohort n=385

HLA-C				
Allele	**AF**	**GF (%)**	**Homozygotes**	**Heterozygotes**
C*0102	0.021	3.9	1	14
C*0102g	0.027	5.5	0	21
C*020202	0.035	7.0	0	27
C*0302	0.008	1.3	1	4
C*030304	0.001	0.3	0	1
C*0303g	0.061	11.9	1	45
C*0304	0.065	12.5	2	46
C*0401g	0.070	13.2	3	48
C*0403	0.004	0.8	0	3
C*0501g	0.084	16.1	3	59
C*0602	0.083	16.1	2	60
C*0701g	0.166	30.9	9	110
C*0702g	0.152	27.5	11	95
C*0704g	0.013	2.6	0	10
C*0726	0.001	0.3	0	1
C*080101	0.005	1.0	0	4
C*0801g	0.009	1.6	1	5
C*0802	0.030	6.0	0	23
C*080301	0.001	0.3	0	1
C*0804	0.001	0.3	0	1
C*1202	0.018	3.4	1	12
C*1203	0.043	8.6	0	33
C*140201	0.010	2.1	0	8
C*1502g	0.040	8.1	0	31
C*1505	0.005	1.0	0	4
C*160101	0.036	7.3	0	28
C*1602	0.004	0.8	0	3
C*1701g	0.003	0.5	0	2
C*1801g	0.001	0.3	0	1

Table A.4 Distribution of HLA-DRB alleles in the HLA-typed cohort n=385

HLA-DRB				
Allele	AF	GF (%)	Homozygotes	Heterozygotes
DRB1*010101	0.076	14.3	3	52
DRB1*010201	0.008	1.6	0	6
DRB1*0103	0.014	2.9	0	11
DRB1*030101	0.128	23.9	6	86
DRB1*0306	0.001	0.3	0	1
DRB1*040101	0.098	18.2	5	65
DRB1*0402	0.009	1.8	0	7
DRB1*040301	0.016	3.1	0	12
DRB1*0404	0.043	8.6	0	33
DRB1*040501	0.009	1.6	1	5
DRB1*0406	0.001	0.3	0	1
DRB1*040701	0.009	1.8	0	7
DRB1*0408	0.004	0.8	0	3
DRB1*070101	0.145	26.2	10	91
DRB1*0801	0.012	2.3	0	9
DRB1*080302	0.004	0.8	0	3
DRB1*090102	0.022	3.9	2	13
DRB1*100101	0.009	1.8	0	7
DRB1*1101	0.023	4.4	1	16
DRB1*110101	0.012	2.3	0	9
DRB1*110201	0.003	0.5	0	2
DRB1*1103	0.007	1.3	0	5
DRB1*110401	0.010	2.1	0	8
DRB1*110601	0.004	0.8	0	3
DRB1*111402	0.001	0.3	0	1
DRB1*1201g	0.010	2.1	0	8
DRB1*120201	0.012	2.3	0	9
DRB1*130101	0.043	8.6	0	33
DRB1*130201	0.038	7.0	2	25
DRB1*130301	0.005	1.0	0	4
DRB1*130501	0.003	0.5	0	2
DRB1*1401g	0.022	3.9	2	13
DRB1*1404	0.013	2.3	1	8
DRB1*140501	0.001	0.3	0	1
DRB1*1501	0.001	0.3	0	1
DRB1*150101	0.156	28.6	10	100
DRB1*150201	0.016	3.1	0	12
DRB1*150202	0.001	0.3	0	1
DRB1*1503	0.001	0.3	0	1
DRB1*150301	0.001	0.3	0	1
DRB1*160101	0.007	1.3	0	5
DRB1*160201	0.003	0.5	0	2

Table A.5 Distribution of HLA-DQB alleles in the HLA-typed cohort n=385

HLA-DQB				
Allele	**AF**	**GF (%)**	**Homozygotes**	**Heterozygotes**
DQB1*050101	0.001	0.3	0	1
DQB1*0201g	0.239	42.3	21	142
DQB1*0301g	0.155	28.1	11	97
DQB1*030201	0.112	20.8	6	74
DQB1*030302	0.060	11.4	2	42
DQB1*0304	0.003	0.5	0	2
DQB1*030501	0.001	0.3	0	1
DQB1*0401	0.005	0.8	1	2
DQB1*0402	0.014	2.9	0	11
DQB1*050101	0.105	19.5	6	69
DQB1*050201	0.013	2.6	0	10
DQB1*050301	0.035	6.5	2	23
DQB1*0504	0.001	0.3	0	1
DQB1*0601	0.030	6.0	0	23
DQB1*060201	0.138	25.5	8	90
DQB1*060301	0.049	9.9	0	38
DQB1*0604g	0.023	4.7	0	18
DQB1*060801	0.001	0.3	0	1
DQB1*0609	0.014	2.6	1	9

Table A.6 NanoString gene expression changes following 2h abacavir treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Abacavir		Fold	Control		Abacavir		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL4	134	108	98	75	**-1.36**	563	664	182	177	**-3.10**
CCL5	1101	1064	1095	1073	**-1.01**	707	321	502	262	**-1.41**
CCL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR1	156	25	80	40	**-1.96**	40	45	18	16	**-2.19**
CCR2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR5	34	40	nd	-	**-**	nd	-	nd	-	**-**
CD4	91	28	128	82	**1.41**	58	53	47	42	**-1.23**
CD40	109	129	46	35	**-2.37**	nd	-	nd	-	**-**
CD40LG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CD80	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CSF2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CTLA4	48	15	42	25	**-1.15**	61	48	51	42	**-1.18**
CX3CL1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CXCR3	120	138	75	79	**-1.60**	60	45	43	34	**-1.42**
CXCR4	5553	4741	4774	2996	**-1.16**	3489	3526	4156	4275	**1.19**
GATA3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
GUSB	70	26	83	46	**1.19**	43	48	44	49	**1.02**
HPRT1	104	84	81	48	**-1.29**	46	49	57	59	**1.23**
ICOS	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IFNG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL10	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12B	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12RB2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL13	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL15	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL17A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18R1	45	30	35	5	**-1.29**	57	74	50	62	**-1.13**
IL1R1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4R	457	416	342	240	**-1.34**	367	482	343	430	**-1.07**
IL5	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL6	nd	-	nd	-	**-**	31	41	48	65	**1.55**
IL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL8	5521	2957	4067	2057	**-1.36**	5909	6958	5258	6382	**-1.12**
IL9	nd	-	nd	-	**-**	nd	-	nd	-	**-**
INHBA	nd	-	nd	-	**-**	nd	-	nd	-	**-**
LAG3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
RPL19	13266	6354	13869	7420	**1.05**	9887	8528	7777	7078	**-1.27**
TNFRSF9	nd	-	nd	-	**-**	nd	-	nd	-	**-**

nd = not detected, SD= standard deviation

Table A.7 NanoString gene expression changes following 2h abacavir treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Abacavir		Fold	Control		Abacavir		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL4	134	108	98	75	**-1.36**	563	664	182	177	**-3.10**
CCL5	1101	1064	1095	1073	**-1.01**	707	321	502	262	**-1.41**
CCL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR1	156	25	80	40	**-1.96**	40	45	18	16	**-2.19**
CCR2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR5	34	40	nd	-	**-**	nd	-	nd	-	**-**
CD4	91	28	128	82	**1.41**	58	53	47	42	**-1.23**
CD40	109	129	46	35	**-2.37**	nd	-	nd	-	**-**
CD40LG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CD80	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CSF2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CTLA4	48	15	42	25	**-1.15**	61	48	51	42	**-1.18**
CX3CL1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CXCR3	120	138	75	79	**-1.60**	60	45	43	34	**-1.42**
CXCR4	5553	4741	4774	2996	**-1.16**	3489	3526	4156	4275	**1.19**
GATA3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
GUSB	70	26	83	46	**1.19**	43	48	44	49	**1.02**
HPRT1	104	84	81	48	**-1.29**	46	49	57	59	**1.23**
ICOS	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IFNG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL10	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12B	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12RB2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL13	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL15	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL17A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18R1	45	30	35	5	**-1.29**	57	74	50	62	**-1.13**
IL1R1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4R	457	416	342	240	**-1.34**	367	482	343	430	**-1.07**
IL5	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL6	nd	-	nd	-	**-**	31	41	48	65	**1.55**
IL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL8	5521	2957	4067	2057	**-1.36**	5909	6958	5258	6382	**-1.12**
IL9	nd	-	nd	-	**-**	nd	-	nd	-	**-**
INHBA	nd	-	nd	-	**-**	nd	-	nd	-	**-**
LAG3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
RPL19	13266	6354	13869	7420	**1.05**	9887	8528	7777	7078	**-1.27**
TNFRSF9	nd	-	nd	-	**-**	nd	-	nd	-	**-**

Table A.8 NanoString gene expression changes following 2h flucloxacillin treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Flucloxacillin		Fold	Control		Flucloxacillin		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL4	134	108	217	241	**1.62**	563	664	405.1	501	**-1.39**
CCL5	1101	1064	964	902	**-1.10**	707	321	507.4	187	**-1.39**
CCL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR1	156	25	50	17	**-3.11**	40	45	25.5	23	**-1.56**
CCR2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR5	34	40	nd	-	**-**	nd	-	nd	-	**-**
CD4	91	28	147	116	**1.61**	58	53	57.5	43	**-1.01**
CD40	109	129	23	14	**-4.73**	nd	-	nd	-	**-**
CD40LG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CD80	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CSF2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CTLA4	48	15	57	36	**1.17**	61	48	54.9	50	**-1.10**
CX3CL1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CXCR3	120	138	72	38	**-1.66**	60	45	47.1	32	**-1.28**
CXCR4	5553	4741	2441	756	**-2.28**	3489	3526	2326.2	2754	**-1.50**
GATA3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
GUSB	70	26	135	126	**1.95**	43	48	51.4	55	**1.19**
HPRT1	104	84	46	11	**-2.29**	46	49	44.5	48	**-1.04**
ICOS	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IFNG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL10	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12B	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12RB2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL13	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL15	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL17A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18R1	45	30	36	11	**-1.24**	57	74	45.4	55	**-1.25**
IL1R1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4R	457	416	273	142	**-1.68**	367	482	354.4	476	**-1.04**
IL5	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL6	nd	-	nd	-	**-**	31	41	38.3	59	**1.24**
IL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL8	5521	2957	2967	1882	**-1.86**	5909	6958	4289.6	4693	**-1.38**
IL9	nd	-	nd	-	**-**	nd	-	nd	-	**-**
INHBA	nd	-	nd	-	**-**	nd	-	nd	-	**-**
LAG3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
RPL19	13266	6354	16976	10744	**1.28**	9887	8528	8501.7	7824	**-1.16**
TNFRSF9	nd	-	nd	-	**-**	nd	-	nd	-	**-**

Table A.9 NanoString gene expression changes following 2h tetanus toxoid treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Tetanus		Fold	Control		Tetanus		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL4	134	108	162	108	**1.22**	563	664	619	761	**1.10**
CCL5	1101	1064	983	1064	**-1.12**	707	321	538	333	**-1.31**
CCL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR1	156	25	146	25	**-1.07**	40	45	45	57	**1.13**
CCR2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR5	34	40	nd	-	**-**	nd	-	nd	-	**-**
CD4	91	28	91	28	**-1.00**	58	53	59	57	**1.02**
CD40	109	129	33	129	**-3.29**	nd	-	nd	-	**-**
CD40LG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CD80	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CSF2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CTLA4	48	15	51	15	**1.05**	61	48	63	61	**1.03**
CX3CL1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CXCR3	120	138	73	138	**-1.64**	60	45	60	62	**1.00**
CXCR4	5553	4741	3131	4741	**-1.77**	3489	3526	3203	3283	**-1.10**
GATA3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
GUSB	70	26	71	26	**1.02**	43	48	42	48	**-1.03**
HPRT1	104	84	100	84	**-1.04**	46	49	54	54	**1.16**
ICOS	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IFNG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL10	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12B	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12RB2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL13	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL15	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL17A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18R1	45	30	49	30	**1.08**	57	74	54	75	**-1.06**
IL1R1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4R	457	416	207	416	**-2.20**	367	482	341	442	**-1.08**
IL5	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL6	nd	-	nd	-	**-**	31	41	36	54	**1.17**
IL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL8	5521	2957	4720	2957	**-1.17**	5909	6958	6704	8421	**1.13**
IL9	nd	-	nd	-	**-**	nd	-	nd	-	**-**
INHBA	nd	-	nd	-	**-**	nd	-	nd	-	**-**
LAG3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
RPL19	13266	6354	13123	6354	**-1.01**	9887	8528	9042	7614	**-1.09**
TNFRSF9	nd	-	nd	-	**-**	nd	-	nd	-	**-**

Table A.10 NanoString gene expression changes following 24h abacavir treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Abacavir		Fold	Control		Abacavir		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL3	455	28	1757	36	**3.86**	28	34	36	49	**1.30**
CCL4	1107	267	1805	236	**1.63**	267	69	236	163	**-1.13**
CCL5	939	600	972	562	**1.04**	600	494	562	412	**-1.07**
CCL7	327	23	208	36	**-1.57**	nd	-	nd	-	**-**
CCR1	235	44	137	48	**-1.72**	44	43	48	44	**1.09**
CCR2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR5	74	19	61	12	**-1.21**	nd	-	nd	-	**-**
CD4	71	37	47	36	**-1.50**	37	46	36	37	**-1.01**
CD40	68	30	106	19	**1.56**	nd	-	nd	-	**-**
CD40LG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CD80	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CSF2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CTLA4	98	67	137	79	**1.41**	67	64	79	78	**1.18**
CX3CL1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CXCR3	41	41	101	38	**2.46**	41	33	38	36	**-1.07**
CXCR4	2100	1897	3258	1781	**1.55**	1897	1560	1781	1534	**-1.06**
GATA3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
GUSB	87	48	106	46	**1.22**	48	52	46	48	**-1.04**
HPRT1	100	55	86	60	**-1.17**	55	52	60	58	**1.09**
ICOS	40	21	31	16	**-1.28**	nd	-	nd	-	**-**
IFNG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL10	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12B	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12RB2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL13	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL15	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL17A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18R1	47	44	66	50	**1.40**	44	61	50	63	**1.15**
IL1R1	35	15	112	15	**3.20**	nd	-	nd	-	**-**
IL2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4R	130	102	213	79	**1.64**	102	92	79	82	**-1.30**
IL5	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL6	156	28	1497	84	**9.60**	28	45	84	139	**2.98**
IL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL8	11475	2634	42123	4215	**3.67**	2634	2646	4215	3453	**1.60**
IL9	nd	-	nd	-	**-**	nd	-	nd	-	**-**
INHBA	nd	-	nd	-	**-**	nd	-	nd	-	**-**
LAG3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
RPL19	10791	7279	10990	7206	**1.02**	7279	7620	7206	7517	**-1.01**
TNFRSF9	nd	-	nd	-	**-**	nd	-	nd	-	**-**

Table A.11 NanoString gene expression changes following 24h flucloxacillin treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Flucloxacillin		Fold	Control		Flucloxacillin		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL3	455	28	134	102	**-3.39**	28	34	63	62	**2.26**
CCL4	1107	267	1437	2002	**1.30**	267	69	489	272	**1.83**
CCL5	939	600	824	498	**-1.14**	600	494	568	253	**-1.06**
CCL7	327	23	102	140	**-3.19**	nd	-	nd	-	**-**
CCR1	235	44	87	70	**-2.70**	44	43	28	3	**-1.56**
CCR2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR5	74	19	92	118	**1.25**	nd	-	nd	-	**-**
CD4	71	37	46	22	**-1.53**	37	46	31	38	**-1.19**
CD40	68	30	22	13	**-3.12**	nd	-	nd	-	**-**
CD40LG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CD80	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CSF2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CTLA4	98	67	134	36	**1.38**	67	64	54	68	**-1.26**
CX3CL1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CXCR3	41	41	48	31	**1.17**	41	33	34	16	**-1.21**
CXCR4	2100	1897	2926	3759	**1.39**	1897	1560	927	712	**-2.05**
GATA3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
GUSB	87	48	74	31	**-1.16**	48	52	45	49	**-1.06**
HPRT1	100	55	115	102	**1.14**	55	52	52	38	**-1.05**
ICOS	40	21	41	55	**1.02**	nd	-	nd	-	**-**
IFNG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL10	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12B	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12RB2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL13	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL15	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL17A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18R1	47	44	79	68	**1.68**	44	61	36	37	**-1.20**
IL1R1	35	15	66	80	**1.87**	nd	-	nd	-	**-**
IL2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4R	130	102	150	133	**1.15**	102	92	52	48	**-1.98**
IL5	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL6	156	28	211	351	**1.35**	28	45	118	191	**4.18**
IL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL8	11475	2634	16269	13533	**1.42**	2634	2646	4918	4653	**1.87**
IL9	nd	-	nd	-	**-**	nd	-	nd	-	**-**
INHBA	nd	-	nd	-	**-**	nd	-	nd	-	**-**
LAG3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
RPL19	10791	7279	11686	4656	**1.08**	7279	7620	8439	10365	**1.16**
TNFRSF9	nd	-	nd	-	**-**	nd	-	nd	-	**-**

Table A.12 NanoString gene expression changes following 24h tetanus toxoid treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Tetanus		Fold	Control		Tetanus		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL3	455	28	645	576	**1.42**	28	34	117	64	**4.21**
CCL4	1107	267	1773	1188	**1.60**	267	69	1488	1732	**5.57**
CCL5	939	600	633	427	**-1.48**	600	494	444	287	**-1.35**
CCL7	327	23	383	117	**1.17**	nd	-	232	179	**-**
CCR1	235	44	261	176	**1.11**	44	43	74	67	**1.68**
CCR2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR5	74	19	173	169	**2.35**	nd	-	nd	-	**-**
CD4	71	37	36	15	**-1.97**	nd	-	nd	-	**-**
CD40	68	30	124	29	**1.82**	nd	-	43	10	**-**
CD40LG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CD80	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CSF2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CTLA4	98	67	136	59	**1.39**	67	64	66	73	**-1.01**
CX3CL1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CXCR3	41	41	58	41	**1.41**	41	33	38	34	**-1.06**
CXCR4	2100	1897	1677	1764	**-1.25**	1897	1560	841	257	**0.44**
GATA3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
GUSB	87	48	75	44	**-1.16**	48	52	55	75	**1.15**
HPRT1	100	55	135	98	**1.35**	55	52	47	22	**0.86**
ICOS	40	21	37	43	**-1.08**	nd	-	nd	-	**-**
IFNG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL10	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12B	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12RB2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL13	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL15	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL17A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18R1	47	44	70	66	**1.48**	44	61	40	42	**0.92**
IL1R1	35	15	39	53	**1.11**	nd	-	nd	-	**-**
IL2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4R	130	102	260	227	**2.00**	102	92	117	105	**1.14**
IL5	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL6	156	28	386	381	**2.47**	28	45	65	54	**2.29**
IL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL8	11475	2634	15481	17044	**1.35**	2634	2646	3079	2771	**1.17**
IL9	nd	-	nd	-	**-**	nd	-	nd	-	**-**
INHBA	nd	-	nd	-	**-**	nd	-	58	70	**-**
LAG3	nd	-	nd	-	**-**	nd	-	34	28	**-**
RPL19	10791	7279	9307	3426	**-1.16**	7279	7620	8648	9415	**1.19**
TNFRSF9	nd	-	nd	-	**-**	nd	-	nd	-	**-**

Table A.13 NanoString gene expression changes following 72h abacavir treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Abacavir		Fold	Control		Abacavir		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCL3	57	64	88	78	**1.53**	nd	-	nd	-	**-**
CCL4	324	212	516	488	**1.59**	413	532	353	462	**-1.17**
CCL5	520	366	652	571	**1.26**	541	380	504	378	**-1.07**
CCL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR1	51	42	43	11	**-1.18**	nd	-	nd	-	**-**
CCR2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CCR5	42	60	27	29	**-1.54**	nd	-	nd	-	**-**
CD4	78	45	70	39	**-1.12**	63	77	46	57	**-1.37**
CD40	53	18	53	19	**-1.01**	nd	-	nd	-	**-**
CD40LG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CD80	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CSF2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CTLA4	84	21	102	26	**1.21**	41	51	39	41	**-1.06**
CX3CL1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
CXCR3	45	16	48	17	**1.06**	30	30	23	21	**-1.30**
CXCR4	1841	1607	1834	1496	**1.00**	601	229	730	410	**1.21**
GATA3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
GUSB	83	27	76	32	**-1.01**	62	81	56	69	**-1.12**
HPRT1	98	79	97	71	**-1.01**	37	15	41	18	**1.10**
ICOS	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IFNG	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL10	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12B	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL12RB2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL13	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL15	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL17A	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL18R1	56	57	81	87	**1.43**	nd	-	nd	-	**-**
IL1R1	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL2	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL4R	210	74	184	60	**-1.14**	84	69	70	69	**-1.21**
IL5	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL6	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL7	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL8	3013	2583	3524	2081	**1.17**	819	945	957	1033	**1.17**
IL9	nd	-	nd	-	**-**	nd	-	nd	-	**-**
INHBA	nd	-	nd	-	**-**	nd	-	nd	-	**-**
LAG3	nd	-	nd	-	**-**	nd	-	nd	-	**-**
RPL19	11803	6376	12823	6747	**1.09**	9705	11841	9767	12299	**1.01**
TNFRSF9	nd	-	nd	-	**-**	nd	-	nd	-	**-**

Table A.14 NanoString gene expression changes following 72h flucloxacillin treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Flucloxacillin		Fold	Control		Flucloxacillin		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	-	nd	-	nd	-	-
CCL3	57	64	60	52	**1.04**	nd	-	nd	-	**1.79**
CCL4	324	212	279	264	**-1.16**	413	532	282	230	**-1.46**
CCL5	520	366	613	509	**1.18**	541	380	503	298	**-1.07**
CCL7	nd	-	nd	-	-	nd	-	nd	-	-
CCR1	51	42	91	103	**1.80**	nd	-	nd	-	-
CCR2	nd	-	nd	-	-	nd	-	nd	-	-
CCR3	nd	-	nd	-	-	nd	-	nd	-	-
CCR4	nd	-	nd	-	-	nd	-	nd	-	-
CCR5	42	60	36	27	**-1.19**	nd	-	nd	-	-
CD4	78	45	56	41	**-1.41**	63	77	34	50	**-1.83**
CD40	53	18	nd	-	-	nd	-	nd	-	-
CD40LG	nd	-	nd	-	-	nd	-	nd	-	-
CD80	nd	-	nd	-	-	nd	-	nd	-	-
CSF2	nd	-	nd	-	-	nd	-	nd	-	-
CTLA4	84	21	75	52	**-1.13**	41	51	37	42	**-1.10**
CX3CL1	nd	-	nd	-	-	nd	-	nd	-	-
CXCR3	45	16	nd	-	**-**	30	30	nd	-	-
CXCR4	1841	1607	1653	1491	**-1.11**	601	229	521	266	**-1.15**
GATA3	nd	-	nd	-	-	nd	-	nd	-	-
GUSB	83	27	68	39	**-1.21**	62	81	41	46	**-1.52**
HPRT1	98	79	110	75	**1.13**	37	15	45	29	**1.20**
ICOS	nd	-	nd	-	**-**	nd	-	nd	-	-
IFNG	nd	-	nd	-	-	nd	-	nd	-	-
IL10	nd	-	nd	-	-	nd	-	nd	-	-
IL12A	nd	-	nd	-	-	nd	-	nd	-	-
IL12B	nd	-	nd	-	-	nd	-	nd	-	-
IL12RB2	nd	-	nd	-	-	nd	-	nd	-	-
IL13	nd	-	nd	-	-	nd	-	nd	-	-
IL15	nd	-	nd	-	-	nd	-	nd	-	-
IL17A	nd	-	nd	-	-	nd	-	nd	-	-
IL18	nd	-	nd	-	-	nd	-	nd	-	-
IL18R1	56	57	73	64	**1.30**	nd	-	nd	-	-
IL1R1	nd	-	nd	-	**-**	nd	-	nd	-	-
IL2	nd	-	nd	-	-	nd	-	nd	-	-
IL4	nd	-	nd	-	-	nd	-	nd	-	-
IL4R	210	74	102	75	**-2.07**	84	69	43	5	**-1.98**
IL5	nd	-	nd	-	-	nd	-	nd	-	-
IL6	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL7	nd	-	nd	-	-	nd	-	nd	-	-
IL8	3013	2583	6714	9810	**2.23**	819	945	2846	2238	**3.48**
IL9	nd	-	nd	-	-	nd	-	nd	-	-
INHBA	nd	-	nd	-	-	nd	-	nd	-	-
LAG3	nd	-	nd	-	-	nd	-	nd	-	-
RPL19	11803	6376	14638	7301	**1.24**	9705	11841	11175	13855	**1.15**
TNFRSF9	nd	-	nd	-	-	nd	-	nd	-	-

Table A.15 NanoString gene expression changes following 72h tetanus toxoid treatment.

	B*57:01 Positive					B*57:01 Negative				
	Control		Tetanus		Fold	Control		Tetanus		Fold
Gene	Average	SD	Average	SD	change	Average	SD	Average	SD	change
CCL11	nd	-	nd	-	-	nd	-	nd	-	-
CCL3	57	64	154	129	**2.69**	nd	-	63	40	**-**
CCL4	324	212	468	327	**1.44**	413	532	466	508	**1.13**
CCL5	520	366	434	356	**-1.20**	541	380	332	210	**-1.63**
CCL7	nd	-	nd	-	-	nd	-	nd	-	-
CCR1	51	42	215	18	**4.24**	nd	-	nd	-	-
CCR2	nd	-	nd	-	-	nd	-	nd	-	-
CCR3	nd	-	nd	-	-	nd	-	nd	-	-
CCR4	nd	-	nd	-	-	nd	-	nd	-	-
CCR5	42	60	135	151	**3.18**	nd	-	nd	-	-
CD4	78	45	122	82	**1.55**	63	77	80	115	**1.28**
CD40	53	18	143	34	**2.67**	nd	-	nd	-	-
CD40LG	nd	-	nd	-	-	nd	-	nd	-	-
CD80	nd	-	nd	-	-	nd	-	nd	-	-
CSF2	nd	-	nd	-	-	nd	-	nd	-	-
CTLA4	84	21	93	39	**1.10**	41	51	71	55	**1.73**
CX3CL1	nd	-	nd	-	-	nd	-	nd	-	-
CXCR3	45	16	33	20	**-1.36**	30	30	29	24	**-1.02**
CXCR4	1841	1607	1339	769	**-1.37**	601	229	425	155	**0.71**
GATA3	nd	-	nd	-	-	nd	-	nd	-	-
GUSB	83	27	84	51	**1.02**	62	81	49	60	**-1.26**
HPRT1	98	79	124	76	**1.26**	37	15	51	37	**1.37**
ICOS	nd	-	nd	-	**-**	nd	-	nd	-	-
IFNG	nd	-	nd	-	-	nd	-	nd	-	-
IL10	nd	-	nd	-	-	nd	-	nd	-	-
IL12A	nd	-	nd	-	-	nd	-	nd	-	-
IL12B	nd	-	nd	-	-	nd	-	nd	-	-
IL12RB2	nd	-	nd	-	-	nd	-	nd	-	-
IL13	nd	-	nd	-	-	nd	-	nd	-	-
IL15	nd	-	nd	-	-	nd	-	nd	-	-
IL17A	nd	-	nd	-	-	nd	-	nd	-	-
IL18	nd	-	nd	-	-	nd	-	nd	-	-
IL18R1	56	57	62	53	**1.09**	nd	-	nd	-	-
IL1R1	nd	-	nd	-	**-**	nd	-	nd	-	-
IL2	nd	-	nd	-	-	nd	-	nd	-	-
IL4	nd	-	nd	-	-	nd	-	nd	-	-
IL4R	210	74	237	99	**1.13**	84	69	86	43	**1.02**
IL5	nd	-	nd	-	-	nd	-	nd	-	-
IL6	nd	-	nd	-	**-**	nd	-	nd	-	**-**
IL7	nd	-	nd	-	-	nd	-	nd	-	-
IL8	3013	2583	2020	1609	**-1.49**	819	945	621	645	**-1.32**
IL9	nd	-	nd	-	-	nd	-	nd	-	-
INHBA	nd	-	nd	-	-	nd	-	nd	-	-
LAG3	nd	-	nd	-	-	nd	-	nd	-	-
RPL19	11803	6376	9117	4351	**-1.29**	9705	11841	7909	8555	**-1.23**
TNFRSF9	nd	-	nd	-	-	nd	-	nd	-	-